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AUXIN AND LEAF FORMATION

BY MARY SNOW AND R. SNOW[1]

(With 17 figures in the text)

I. INTRODUCTION

It has not yet been determined what is the effect of applying an auxin directly to the actual stem apex above the youngest leaf primordia, or to some part of the stem apex. It seemed to us of interest to investigate this question partly for its own sake and partly in the hope of discovering whether auxin is likely to play a part in the normal determination and growth of leaf primordia. While this work was in progress, there appeared an interesting paper by Laibach & Mai (1936), who applied lanoline containing hetero-auxin to the outsides of the outer leaves of the small axillary buds of shoots of tomatoes and of *Coleus thyrsoides*. They decapitated the shoots and renewed the hetero-auxin paste occasionally while the axillary buds grew out. Various abnormalities were observed in the growth of the axis and of the older leaves of the buds, and some of these—namely, abnormal unions between leaves—are similar to some which will be described here,[2] though in the present experiments the hetero-auxin paste was applied to the stem apex, and the unions were mostly between leaf primordia which arose after the auxinations.

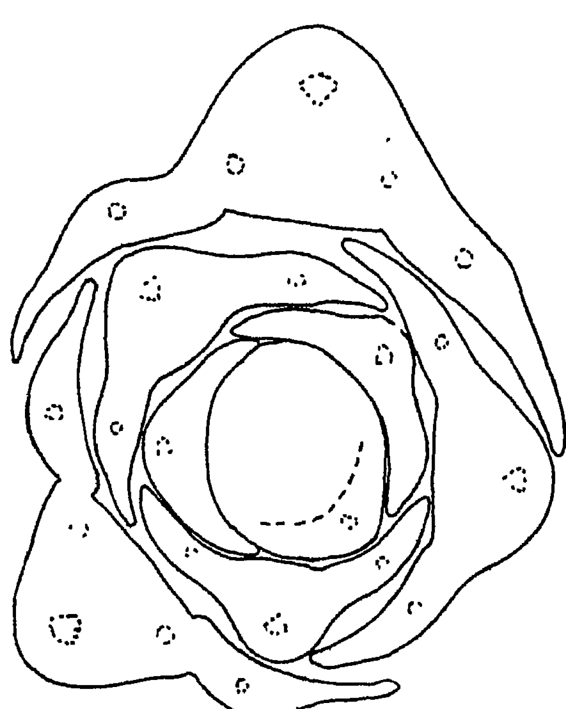
II. MATERIALS, METHODS AND TERMINOLOGY

For our experiments we used seedlings of *Lupinus albus* and plants grown from runners of *Epilobium hirsutum*, two species which we had previously found convenient for operations on stem apices

[1] Fellow of Magdalen College, Oxford.

[2] It is not quite clear whether in the experiments of Laibach & Mai the leaves which were abnormal or united were always amongst those which the paste actually touched. But it seems probable that they were, since the authors state that these leaves were amongst the oldest few leaves of the bud (up to the fourth leaf), and the paste was renewed as the buds grew out.

(Snow & Snow, 1931, 1933, 1935). Transverse sections of normal apices of these species in the vegetative condition are shown in Figs. 1 and 2. The phyllotaxis of *Lupinus albus* is spiral, with a mean divergence angle of $136°\cdot3 \pm 0°\cdot26$, the probable error of a single angle being $1°\cdot88$: the contacts are 1, 2 and 3. The phyllotaxis of *Epilobium hirsutum* is decussate so long as the apices are vegetative, as were those used (see Snow & Snow, 1931, 1935). The lupin apices were also all vegetative at the time of auxination, but some of them began



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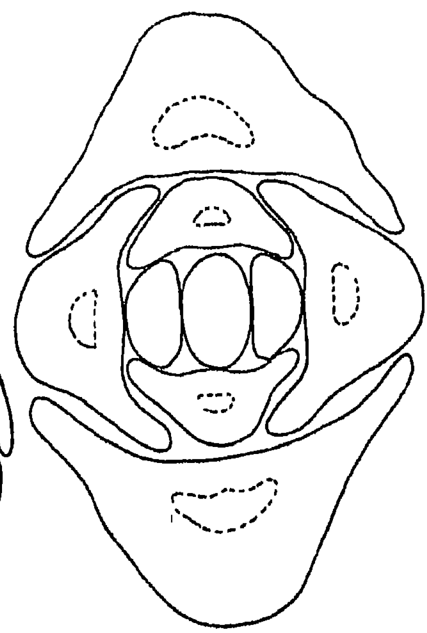

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Fig. 1. Transverse section of normal bud of *Lupinus albus* just below stem apex. ×51.

Fig. 2. Transverse section of normal bud of *Epilobium hirsutum*. ×71.

rather soon afterwards to form bracts, having floral buds in their axils, instead of true leaves. The appearance in transverse section of these bracts and floral buds, which are the beginning of the terminal inflorescence, can be seen in the apex shown in Fig. 7, in which the primordium marked I_3 and all younger primordia are bracts. The last few true leaves that are formed before the bracts in the lupins have vegetative axillary buds, but those formed earlier have no axillary buds. In *Epilobium hirsutum* every leaf has an axillary bud.

In preparing for the experiments, the young leaves which over-arched the stem apex were cut away under a dissecting binocular in the manner described previously, until the stem apex with the very youngest few leaf primordia became visible. Then a tiny spot of

lanoline containing hetero-auxin was carefully placed so as to cover either the whole stem apex above the youngest leaf primordia or that part of the apex from which the next primordium or the next but one was due to arise. Often it could not be avoided that the lanoline touched also the upper faces of the youngest primordia below the parts of the stem apex to which it was applied. The strength of the paste was usually 1 in 2000—that is, a solution of one part of hetero-auxin in 1000 parts of water stirred up with an equal quantity of wool-fat.[1] Sometimes a paste of 1 in 1300 was used, but this, though it did not stop growth, often caused injuries to small groups of cells close to the surface, which appeared dark brown or black. The lupins were mostly auxinated in the autumn of 1935, and the *Epilobiums* in early May 1936. The plants were kept screened from direct sunlight.

At various times after the operations (usually 16 or 17 days) the buds were fixed in 95 per cent alcohol, and subsequently they were embedded in collodion, examined by transverse sections in 66 per cent glycerine and drawn under drawing eyepiece or projection apparatus.

In describing the results, the leaf primordia will be denoted as previously. Those present at the time of operation will be called P_1, P_2 and so on, P_1 being the youngest: those arising after the operation will be called I_1, I_2 and so on, I_1 being the oldest. In *Epilobium hirsutum*, since the leaves are in opposite pairs, there are normally two P_1's, two P_2's and so on. By the stem apex will be meant all the part above the youngest visible primordia. The auxination of that part of the stem apex from which I_1 or I_2 was due to arise will usually be called, for brevity, the auxination of I_1 or I_2, although those primordia were not yet visible at the time. In order to show subsequently which primordium was P_1, a known older leaf, such as P_4 or P_5, was marked by being split at the time of the auxination.

III. Unions between leaf primordia

Quite unexpectedly the most striking effect of the auxinations was to cause unions amongst the leaf primordia that were formed soon after the auxination or immediately before it. Thus in *Lupinus albus* out of sixteen experiments in which the whole stem apex above the youngest primordia was covered with hetero-auxin paste, in four P_1 and I_1 were afterwards found to be united (see Fig. 3*a* and *b*, which shows two sections at different levels through one such apex).

[1] Previously the concentration of a similar paste was called 1 in 1000 (Snow, 1936).

In another five experiments, I_1 and I_2 were united (see Figs. 4 and 13) and in two others I_1 was united both with I_2 and with I_3 (see Figs. 5 and 6). In the apex shown in Fig. 5 I_1 is abnormally large and has two vascular bundles, so that it may be considered as a double leaf. Further unions between still younger leaf primordia can be seen in Figs. 3, 4, 6 and 13. These were probably due to a second lot of auxin paste which was applied to the stem apex a week after the first lot in this group of experiments. In controls pure lanoline placed similarly upon the apex had no effect.

The primordia often united also in other experiments in which the auxin paste was placed only on I_2—that is, on the part of the stem apex from which the next primordium but one was due to arise. Thus out of fifteen such experiments, in five I_1 and I_2 were united (see Figs. 7 and 8), in one I_2 and I_3 were united (see Fig. 9), and in one more I_2 was united both with I_1 and with P_1.

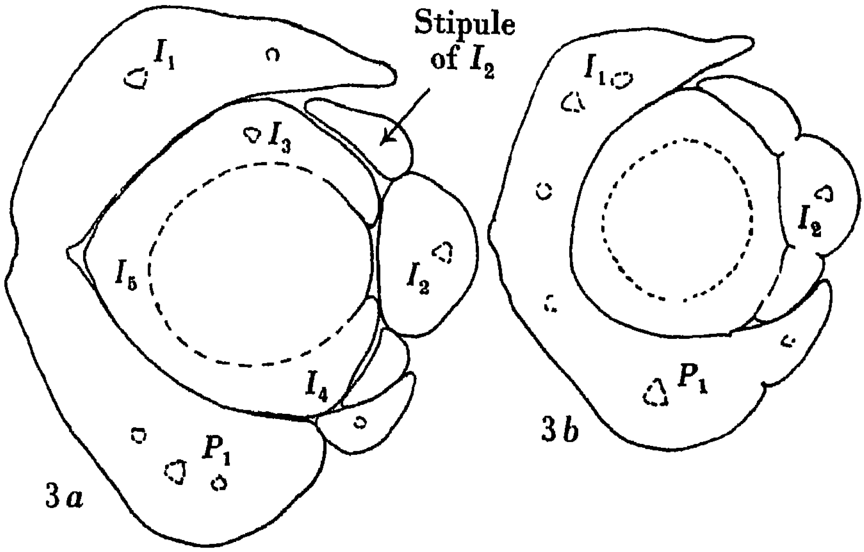
When the auxin paste was placed on the site of I_1, not so many unions of leaf primordia were caused. Thus after seventeen such auxinations there were only four unions, and these were all between I_1 and I_2 (see Figs. 10 and 11). In the auxinations of I_1 and I_2 the paste was applied only once.

Similar experiments on *Epilobium hirsutum* caused a few unions amongst the leaf primordia, though they were less frequent. But this may have been only because the *Epilobiums* were auxinated in early May, when the rather higher temperatures may have caused the little spot of paste to slip off or become inactive more easily. Auxination of the whole stem apex in *Epilobium* caused one union between I_1 and the two P_1's (Fig. 14): auxination of I_1 caused two unions between P_1 and I_1 (Fig. 15), one union between P_1 and P_2, and two unions between P_2 and one or both P_3's: auxination of P_1 caused one union between P_1 and P_2 (Fig. 16). It may be noted that in *Epilobium*, though not in the lupins, P_2 and P_3 were sometimes involved in unions. This was probably because they had some of the paste smeared on to them accidentally.

Some further points concerning the united primordia will be mentioned below, when their interpretation is discussed.

IV. ENLARGEMENT OF AUXINATED PRIMORDIA AND OF THEIR BUDS

In the lupins when the site of I_1 or of I_2 had been auxinated, this primordium was often found to be abnormally large in the radial or tangential directions or in both; it was never abnormally small. Thus



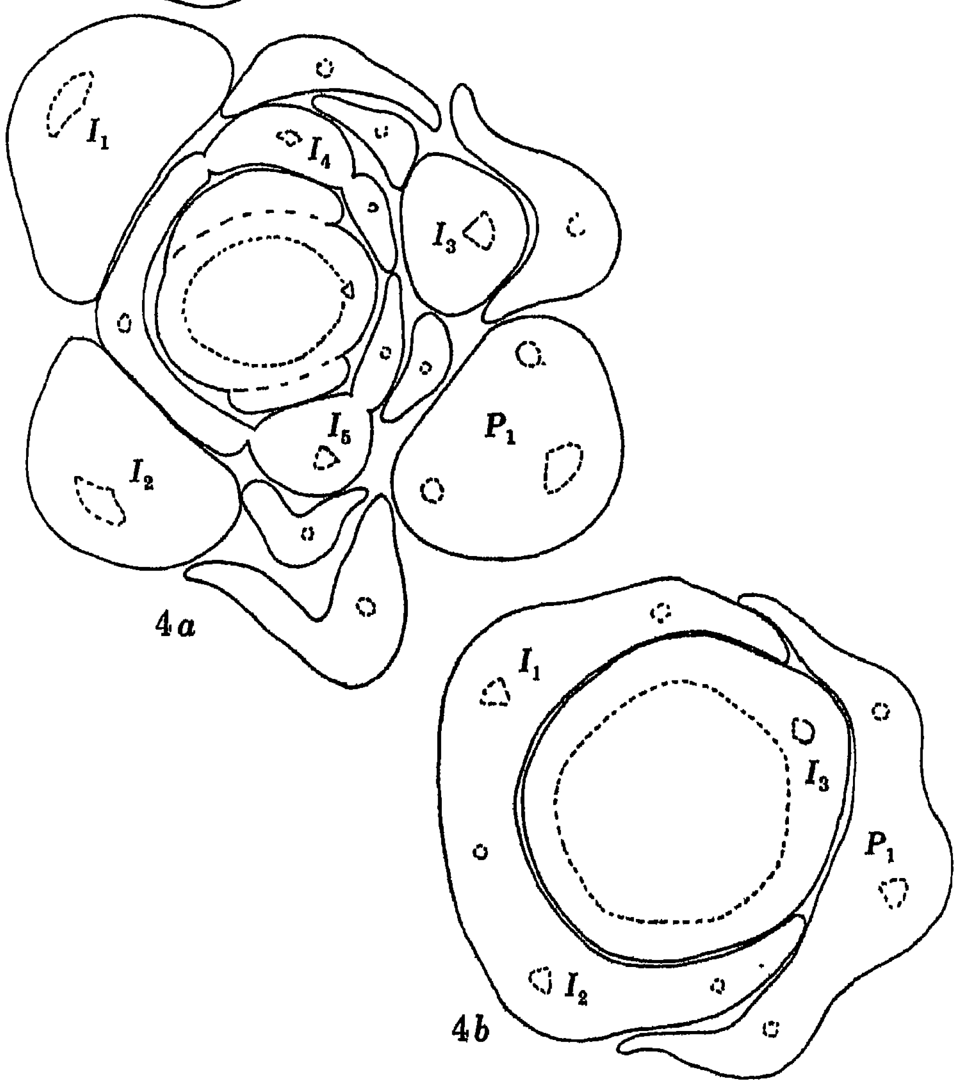
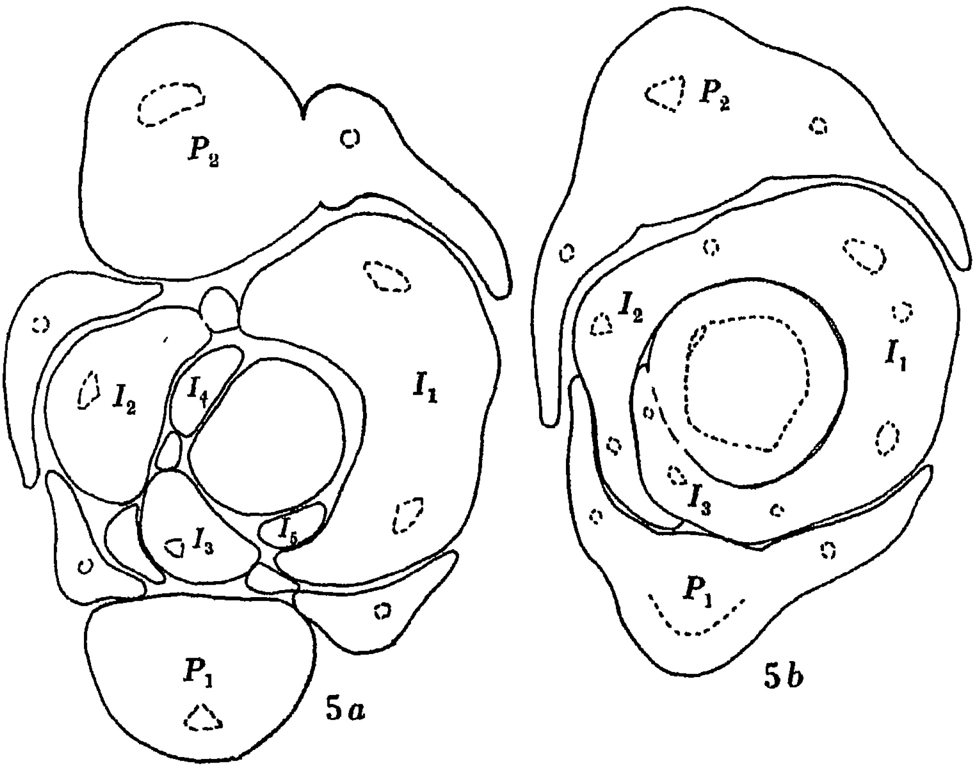


Fig. 3 *a* and *b*. *Lupinus*. Auxin on apex. P_1 and I_1 united, also I_3, I_4 and I_5. *a* and *b* are sections at different levels. $a \times 58$, $b \times 44$.

Fig. 4 *a* and *b*. *Lupinus*. Auxin on apex. I_1 and I_2 united, also I_4 and I_5. $a \times 48$, $b \times 40$.



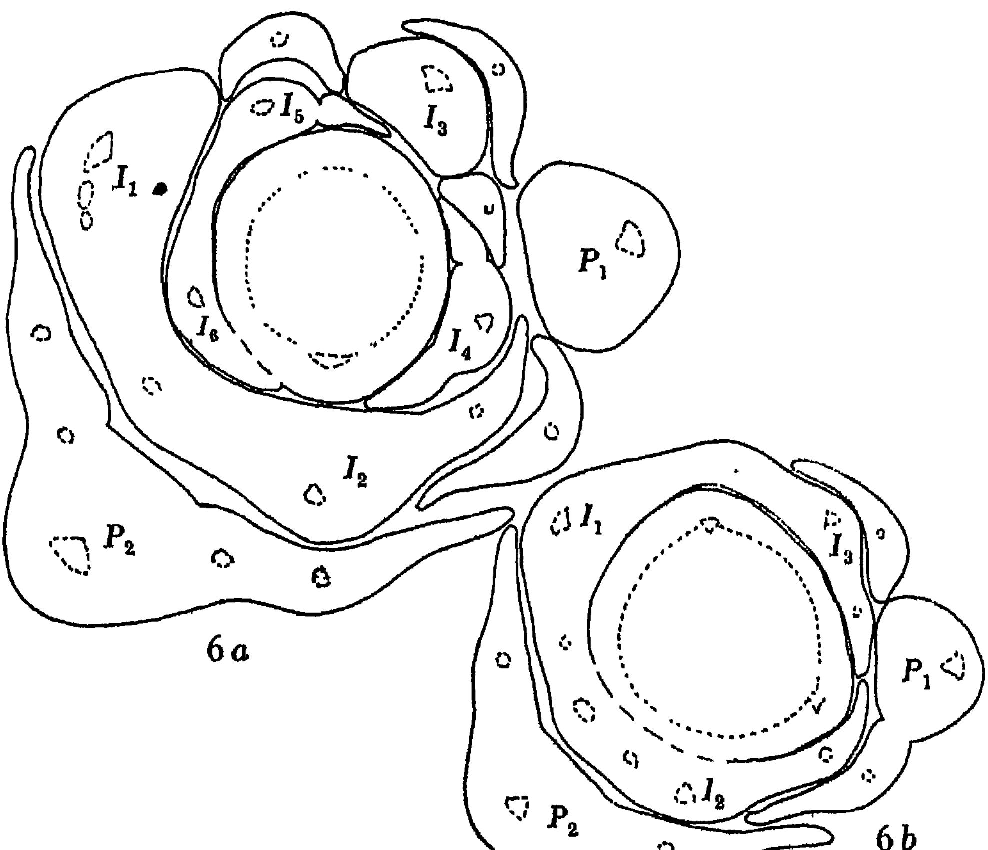


Fig. 5 *a* and *b*. *Lupinus*. Auxin on apex. I_1 united with I_2 and I_3. $a \times 49$, $b \times 46$.

Fig. 6 *a* and *b*. *Lupinus*. Auxin on apex. I_1 united with I_2 and I_3, also I_5 with I_6. $a \times 46$, $b \times 35$.

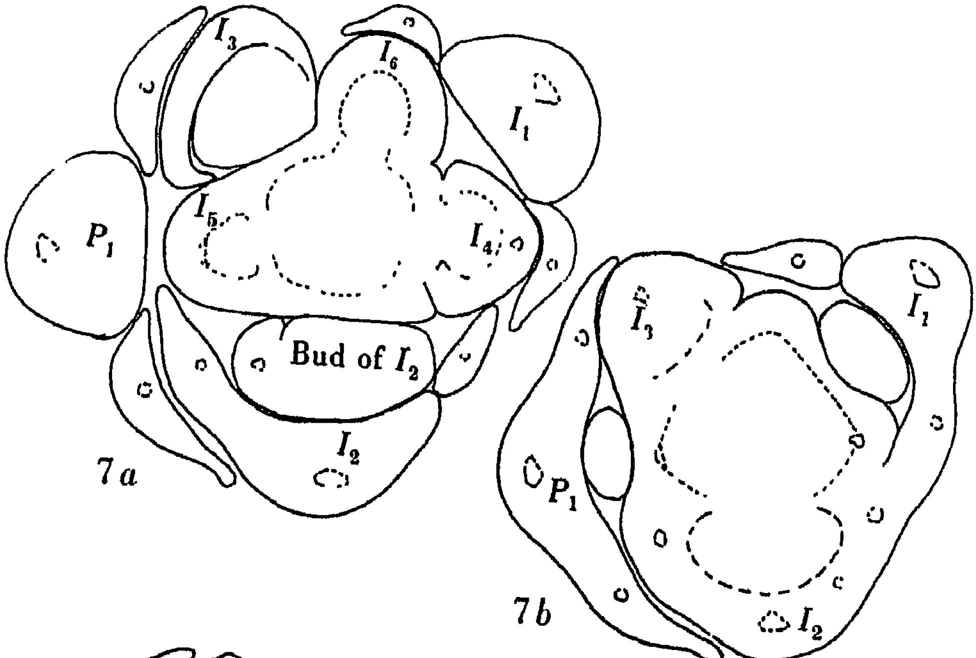


Fig. 7*a* and *b*. *Lupinus*. Auxin on I_2. I_1 and I_2 united. $a \times 48$, $b \times 43$

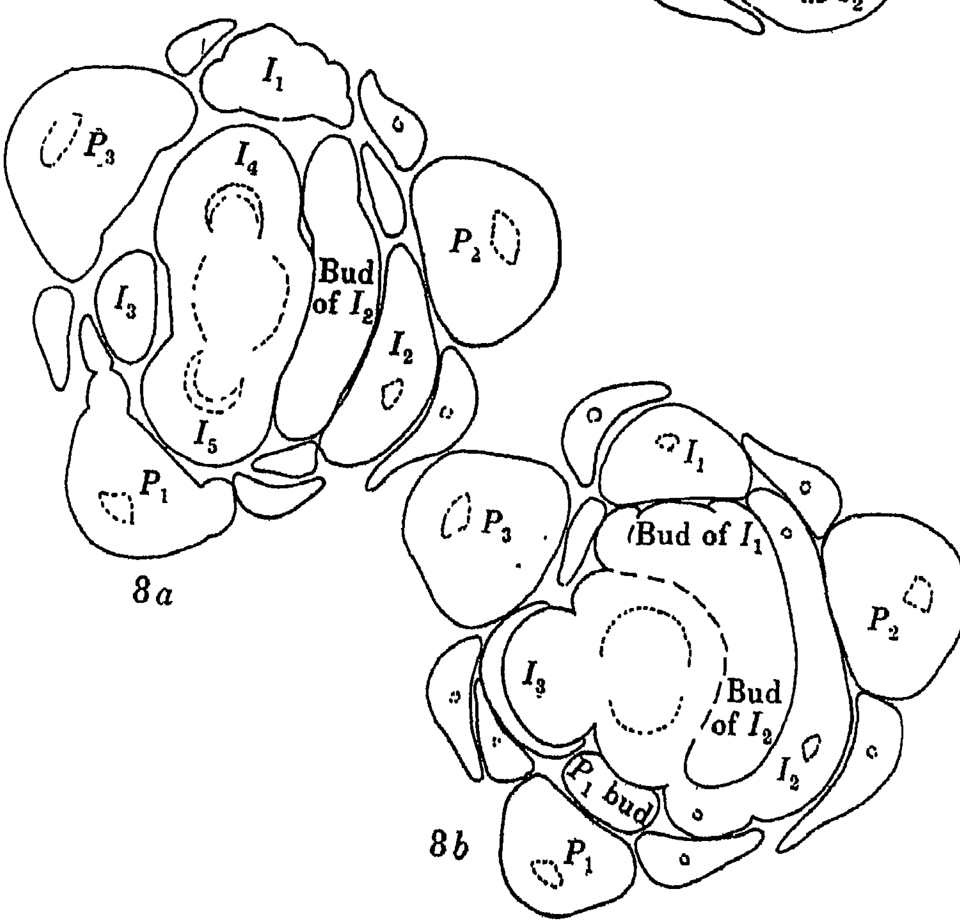
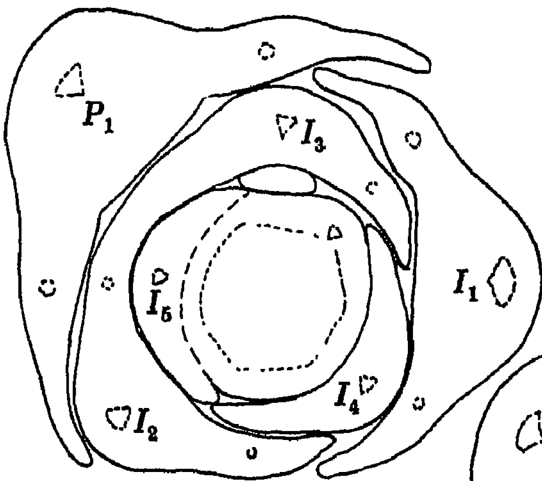
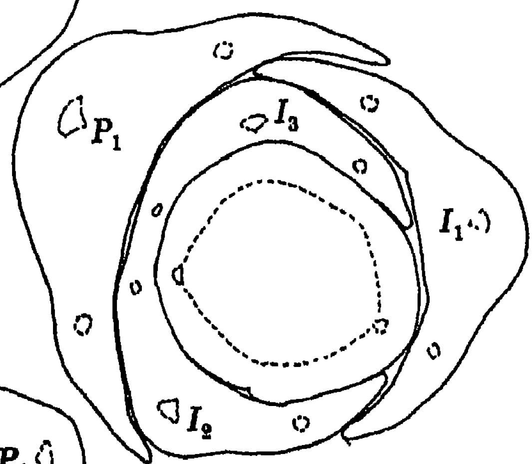


Fig. 8*a* and *b*. *Lupinus*. Auxin on I_2. I_1 and I_2 united, and also their buds. $\times 52$.



9 *a*



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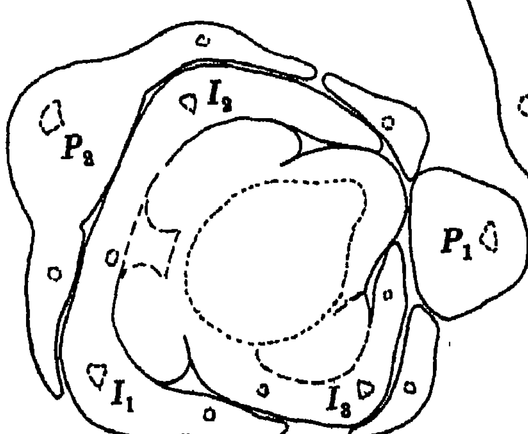

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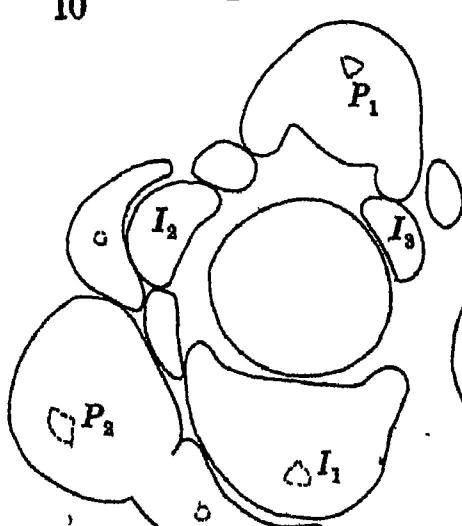
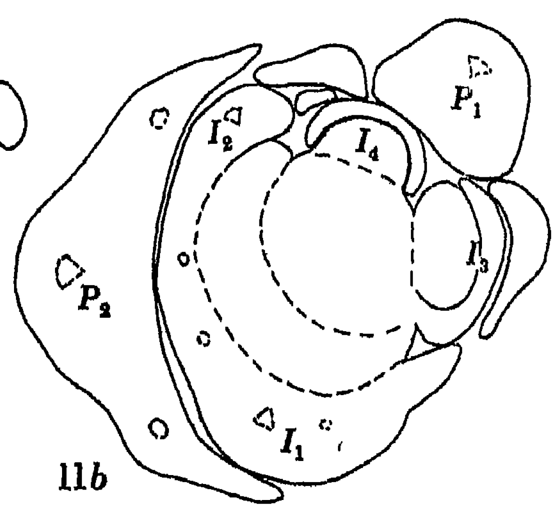


11 *a*

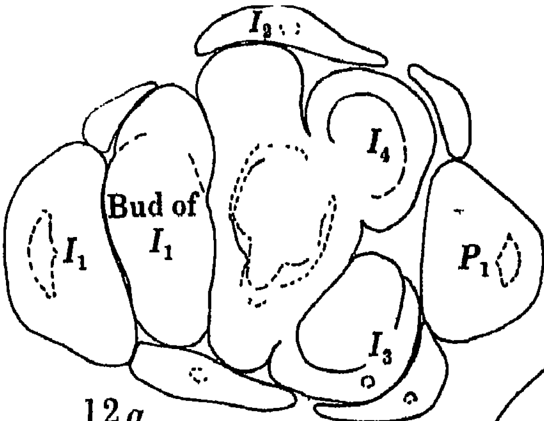


11 *b*

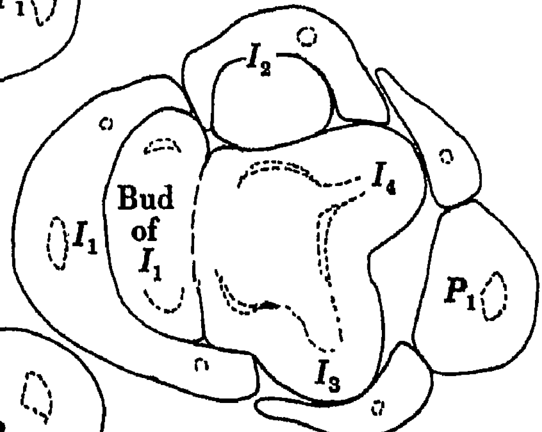
Fig. 9 *a* and *b*. *Lupinus*. Auxin on I_2. I_2 and I_3 united. ×44.

Fig. 10. *Lupinus*. Auxin on I_1. I_1 and I_2 united. ×58.

Fig. 11 *a* and *b*. *Lupinus*. Auxin on I_1. I_1 and I_2 united and also their buds. ×54.



12 *a*



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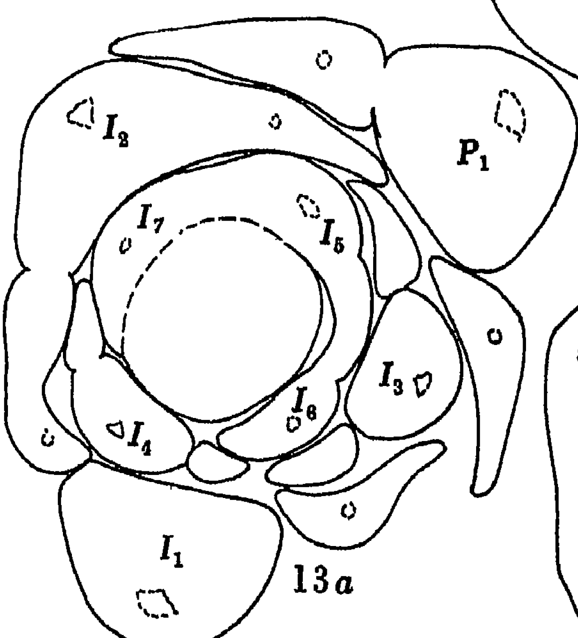
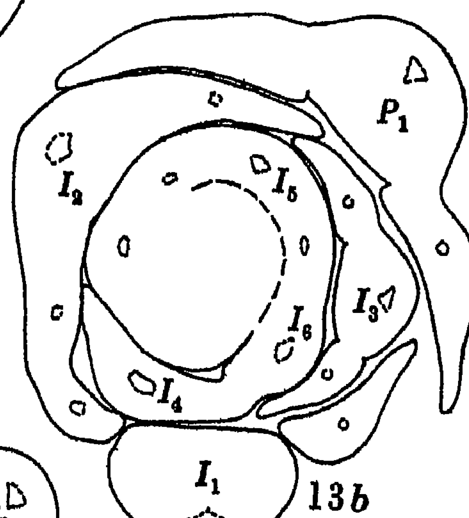
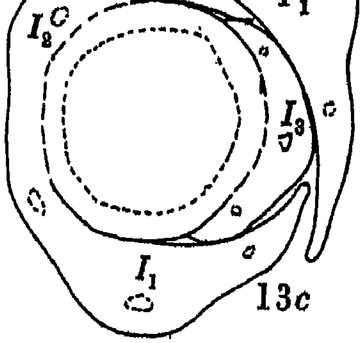


13 *a*



13 *b*



13 *c*

Fig. 12 *a* and *b*. *Lupinus*. Auxin on I_1. I_1 and its bud enlarged. × 54.

Fig. 13 *a*, *b* and *c*. *Lupinus*. Auxin on apex. I_1 and I_2 united, and also I_4 I_6, I_5 and I_7 united. *a* × 54, *b* × 45, *c* × 35.

after seven out of seventeen auxinations of I_1, I_1 was abnormally large (see Figs. 11 and 12), and after nine out of fifteen auxinations of I_2, I_2 was abnormally large (see Figs. 7 and 8). In Fig. 11 it can be seen that I_1, which was auxinated, is larger than P_1, and in Figs. 7 and 8 that I_2, which was auxinated, is larger than I_1.

Also after a few of the auxinations of I_1 or I_2 the auxinated primordium subtended an abnormally large axillary bud. Examples have been shown in Figs. 7, 8 and 12. In Fig. 8 the buds of I_2 and I_1 are united. The leaves subtending these enlarged buds were all amongst the last few true leaves that were formed before the floral bracts, and these last few leaves are regularly the only ones which subtend axillary buds. In other apices in which the auxinated primordium would not normally have subtended an axillary bud, having been formed at an earlier stage of development, it did not appear that the auxin ever caused a bud to form in its axil.

V. DISPLACEMENTS OF PRIMORDIA

It is a question of interest whether one can alter the exact position in which a leaf primordium will be formed by auxinating a part of the stem apex a little to one side of its normal position. And if so, will the primordium be displaced towards the auxinated region or away from it?

In the lupins when I_1 or I_2 was auxinated, it was often noticed that one of the neighbouring primordia was formed with its morphological centre abnormally close to that of the auxinated primordium, as was recognized by measuring the divergence angle between their centres. But these changes of position were not large, except sometimes when the abnormally close primordia were united: and then the abnormal closeness of their centres may have been partly due to the union.

Clearer evidence is provided by the experiments on *Epilobium* in which one of the I_1's was auxinated. For in several of these experiments the P_1's were afterwards found to have been displaced towards the auxinated I_1. An example is shown in Fig. 17, in which the divergence angle between the centres of the P_1's on the auxinated side of the apex is only 155° instead of 180°. The P_1's were never displaced in the opposite direction. It may seem surprising that the P_1's, which were already visible at the time of auxination, were displaced by the auxination: but one must suppose that they were to some extent dedifferentiated and determined afresh.

The auxinated primordia were themselves often displaced a little in one direction or the other, both in *Epilobium* and in *Lupinus*, as was indicated by their divergence angles from older leaves. But this was probably because the auxin paste was sometimes accidentally placed a little to one side or the other of the exact position intended, so that these displacements do not show in which direction the paste tends to displace primordia.

From the facts reported in this section, it appears that the primordia which arise from neighbouring regions tend to be displaced *towards* an auxinated part of the stem apex.

VI. CHANGES IN SUBSEQUENT PHYLLOTAXIS

An *Epilobium* apex in which the stem apex was auxinated and in which the phyllotaxis thereupon became spiral instead of decussate is shown in Fig. 14. It can be seen that one of the I_1's is greatly displaced laterally, and also that it is inserted at a much lower level than the other I_1, as if it had somehow been kept down to a lower level by its union with the P_1's.

The explanation of the displacement of I_1 is probably that the auxin paste was accidentally placed not uniformly all over the apex, but in excess on one side of the part from which I_1 arose. But whatever may be the explanation of the displacement of I_1, the subsequent change of phyllotaxis can readily be explained as a consequence of it, if one proceeds upon the hypothesis, which we have supported previously (1931, 1933, 1935), that each new primordium arises from the first sufficient space which becomes available on the surface of the stem apex between and above the primordia already present. For when the I_1's have arisen in positions that are neither opposite nor at the same level, the apex has become completely asymmetric, and spaces for subsequent primordia become available (through the growth of the stem apex) one at a time. The phyllotaxis therefore necessarily continues asymmetric, each primordium arising (as can be seen) in the first available space between and above the older primordia. Just the same explanation can be applied to the change to spiral phyllotaxis shown in Fig. 16, except that in the apex there illustrated P_1, which was the auxinated primordium, was displaced laterally and was also inserted at an abnormally low level.

In a previous paper on *Epilobium* (1935) we have described numerous changes from decussate to spiral phyllotaxis which were induced by splitting the stem apex longitudinally in a diagonal plane

of the phyllotaxis. The explanation of those changes was the same in principle and was given in greater detail, and we discussed there what conclusions may be drawn. The working hypothesis also was previously stated more exactly (1931, 1935).

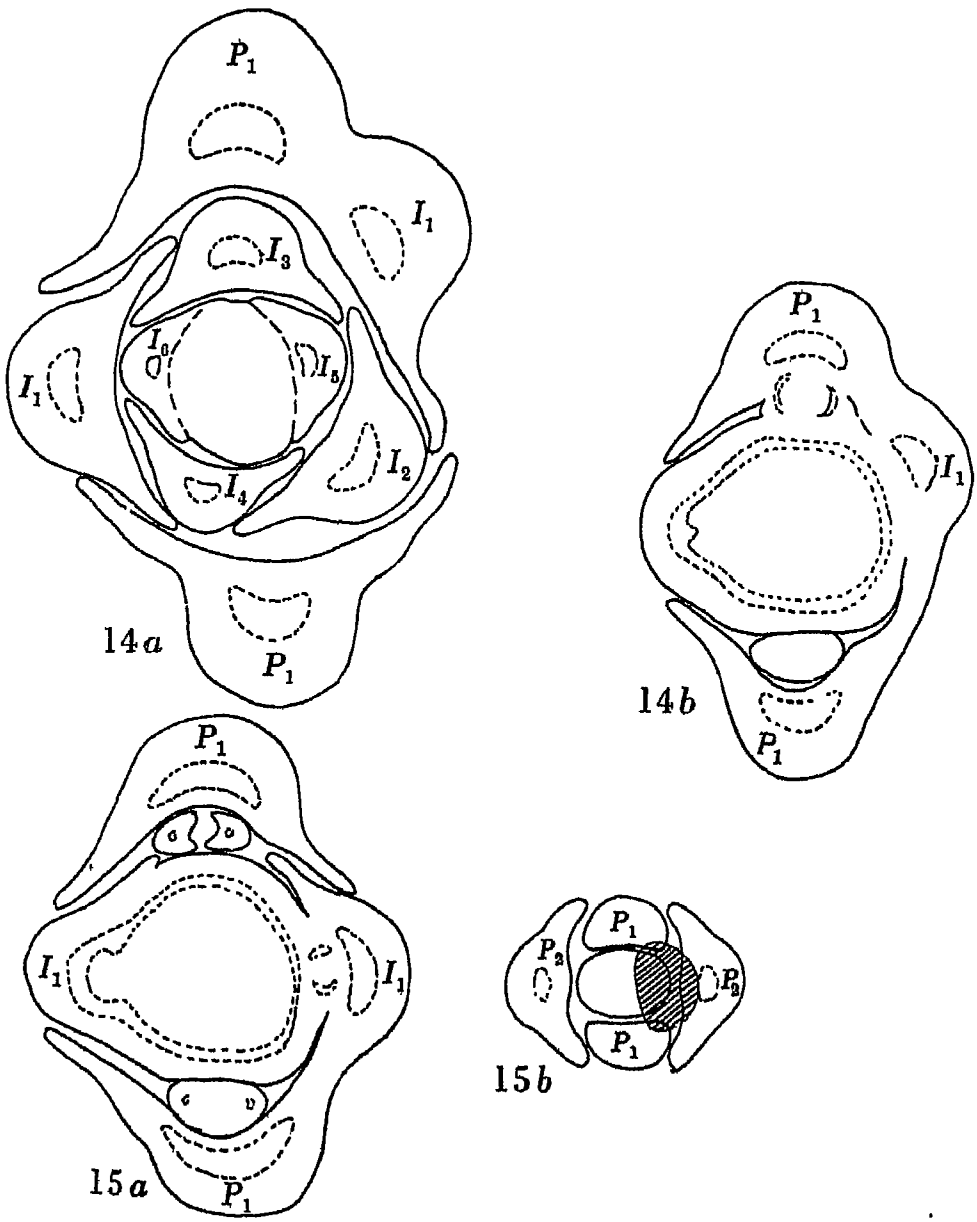


Fig. 14 *a* and *b*. *Epilobium*. Auxin on apex. One I_1 is united with both P_1's, and the phyllotaxis becomes spiral. *a* ×45, *b* ×30.

Fig. 15 *a* and *b*. *Epilobium*. Auxin on one I_1. *a* shows the auxinated I_1 united with P_1. *b* is a diagram showing the original position of the auxin paste as a shaded area. *a* ×26.

In the lupin apices also the phyllotaxis was often considerably altered after the auxinations, but the changes were more complicated and less interesting. This was mainly because in some of the experiments on the lupins (those in which the whole apex was auxinated)

a second lot of auxin paste was usually applied a week after the first lot, while in the others the apices often passed into the flowering condition rather soon after the auxinations.

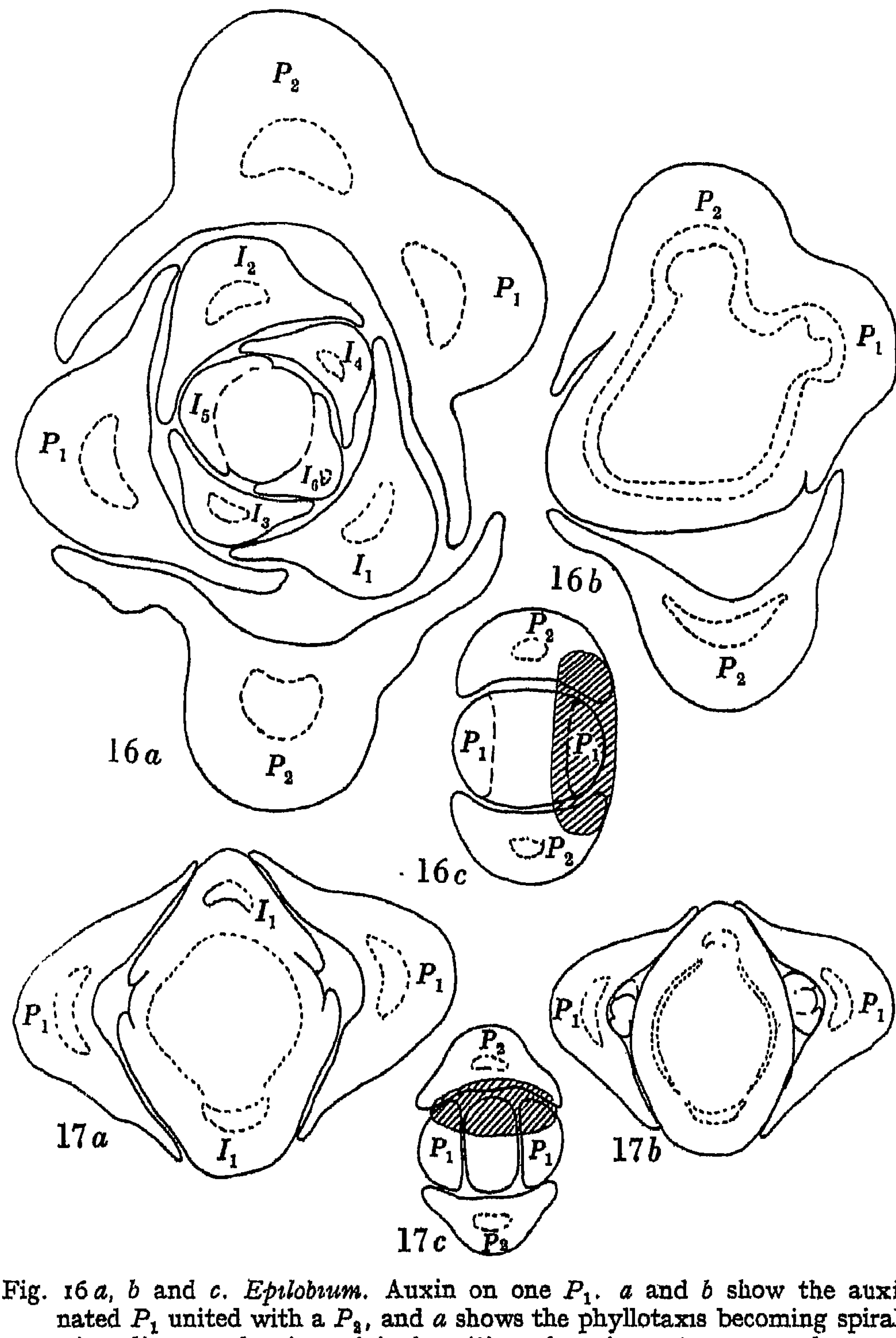


Fig. 16 *a*, *b* and *c*. *Epilobium*. Auxin on one P_1. *a* and *b* show the auxinated P_1 united with a P_2, and *a* shows the phyllotaxis becoming spiral. *c* is a diagram showing original position of auxin paste. *a* × 53, *b* × 35.

Fig. 17 *a*, *b* and *c*. *Epilobium*. Auxin on one I_1. *a* and *b* show the P_1's converging towards the auxinated I_1. *c* is a diagram showing original position of auxin paste. *a* × 34, *b* × 26.

VII. DISCUSSION

It has been shown in section IV that one effect of applying hetero-auxin in lanoline to a part of the stem apex is to cause the leaf primordium and axillary bud which subsequently arises from that part to be abnormally large. It appears therefore that the direct effect of an auxin on bud growth is a promoting one.

Plch (1936, p. 368) has found that when he put hetero-auxin paste (1 in 500) on the free convex surface of the bud in the axil of one of the cotyledons of a decapitated pea seedling, this bud was *retarded* in growth in comparison with the opposite axillary bud. But since he put the paste on the outer surface of the bud (probably on its lowest leaf and basal internode) and not on the stem apex, it is probable that he put it on a region which was morphologically below the parts of the bud which were retarded in growth. Consequently the retardation was probably an effect of the same kind as the retardation of the main shoot which, as Le Fanu (1936) has shown, can be caused in pea seedlings by applying hetero-auxin paste below the elongating zones, although the same paste when applied above these zones promotes growth (see also Snow, 1936). It is hoped to discuss soon in another paper the nature of this inhibiting effect, which may be indirect and is probably of the same nature as correlative inhibition. For the present this at least is clear, that in order to determine with certainty the direct effect of auxin paste on bud growth, one must apply it (in a morphological sense) from above.

Here we must consider in more detail how the auxin paste on the stem apex caused the leaf primordia to be united. It might at first be thought that it did so by enlarging one or more of the united primordia, or by displacing them so that their centres were formed abnormally close to one another. But these changes cannot provide the whole explanation, though they may contribute to it, since without auxin paste the primordia in the two species operated upon can unite only when from some special cause they arise at nearly the same level and therefore nearly simultaneously, as will be pointed out below. When they are at considerably different levels (as were many of the united primordia in the present experiments), then without auxin paste they do not unite, neither in these two species nor (apart from a few exceptions) in other species, not even when they overlap and are in contact, as can be seen in normal apices. Moreover although in the auxinated lupin apices the divergence angle between the centres of united primordia was usually considerably less than

the normal angle of 136°·3, yet it was not always so: for in two apices it was 131° (see Fig. 8), and in two others 136° and 140°.

It appears therefore that an important factor in the bringing about of the unions was that the auxin paste made it possible for primordia at considerably different levels to unite or to arise united. That they did do so can be seen especially clearly in Figs. 5, 6 and 7 for *Lupinus* and in Figs. 14 and 15 for *Epilobium*.

This interpretation fits in well with the explanation offered previously (1933, p. 399) for the unions between leaf primordia in *Lupinus* which followed when certain slight cuts were made in the stem apex. For it was there pointed out that as a result of these operations the spaces which subsequently became available on the stem apex for the formation of primordia were often situated at the same, or nearly the same, level, whereas normally they are not (see 1933, Figs. 18 and 19). When this happened and when the available spaces were close enough together, it was possible for two primordia to be determined over areas of the stem apex which partially coincided: and then the primordia arose simultaneously (or nearly so) and united. Thus without an artificial supply of auxin primordia can arise united only when, for some special reason, they are at nearly the same level: but with such a supply of auxin they can be united even when at different levels.

In the experiments of Laibach & Mai (1936), in which auxin paste was put on the outsides of axillary buds, unions were often formed between the outer leaves, which naturally were already present at the time of auxination. Also in two of the present experiments on *Epilobium*, the primordia P_2 and P_3, which were already present, united, probably because some of the paste was accidentally smeared on to them. But most of the unions in the present experiments were between primordia which were formed after the auxinations, and amongst these united primordia, which were usually of rather different ages, the younger were probably united with the older from the start. For the morphological centres of the united primordia were often so close that there cannot have been room for two complete and separate primordia to arise (see, e.g. Figs. 4 and 6).

There are some indications that it may be a fairly general rule that increased supply of auxin to rudimentary apical parts leads to more union or (as is the same thing) to less division, whereas decrease of auxin leads to less union or more division. For firstly in addition to the unions between leaves already mentioned, Laibach & Mai (1936) after applying hetero-auxin paste to the outer leaves of axillary buds

of tomato plants sometimes found that these leaves, which normally are divided, became entire or nearly entire. Sometimes also they were united abnormally with the stem. On the other hand we have found, after applying hetero-auxin paste to the site of I_1 in a plant of *Dianthus barbatus*, that this primordium developed with two vascular strands at the base and divided into two parts higher up, which is an effect of the opposite kind.

Secondly, Skoog (1935) in a recent interesting paper has shown that X-rays applied to shoots for a few minutes tend to destroy the auxin already present and also, in pea seedlings at least, to prevent the terminal buds and young leaves of the shoot from forming auxin at the normal rate for many days afterwards. Now several observations made by other workers show that treatment of seeds or seedlings with X-rays for short periods sometimes causes the rudimentary apical parts to divide up more actively afterwards. Thus Johnson, after treating seeds or seedlings with X-rays, has observed dichotomies of the main stems, which presumably resulted from dichotomies of the stem apices, in sunflower seedlings, in tomatoes and in *Cosmea* (1926), in *Zinnia elegans* (1936*a*), in *Atriplex hortensis* (1933) and in species of *Impatiens*, *Alonsoa*, *Centaurea* and *Leptosyne* (1936*b*). She has also observed divisions of flower buds in tomatoes leading to the formation of two or three ovaries on one floral axis (1931), and fasciations of the terminal buds in sunflower seedlings, in tomatoes and in *Cosmea* (1926). Also Haskins & Moore (1935) have observed divided leaves instead of the normal entire leaves in *Citrus* seedlings after treatment with X-rays.

On the other hand Johnson (1931) has observed many unions of leaflets with one another or with the petiole in tomatoes after treatment with X-rays, and this is an effect in the opposite sense and so an exception to what appears to be the rule.

There are various other leaf abnormalities which have frequently been found after treatment with X-rays, but which do not indicate either increased or diminished division. These may be due to effects of the X-rays other than the decrease in formation of auxin. The increased outgrowth of axillary buds is probably also irrelevant, though according to Skoog (1935) this is due to diminution of the auxin formed by the terminal bud.

If it should be confirmed that decrease of auxin leads to more division in rudimentary parts, this would support a suggestion implied by Laibach & Mai (1936) that divisions of embryonic parts may be brought about through local accumulations of auxin. For it is

possible that when the supply of auxin is decreased, it may more readily become divided up into local accumulations. This suggestion is also supported to some extent by the facts reported here, which show that auxination of part of a stem apex tends to enlarge the primordium which arises subsequently from that part and to displace towards the auxinated part the primordia which arise from neighbouring regions.

VIII. Summary

1. A paste of lanoline containing hetero-auxin, at a concentration of 1 in 2000 or 1 in 1300, was placed on exposed stem apices of *Lupinus albus* and *Epilobium hirsutum* above the youngest leaf primordia: or sometimes it was placed only on that part of the stem apex from which the next primordium or the next but one was due to arise.

2. As a result the primordia which arose soon afterwards, or immediately before, were often found to be united. Pure lanoline, without hetero-auxin, had no effect.

3. When the paste had been applied only to part of the stem apex, the primordium which subsequently arose from that part was often abnormally large, and so also was its axillary bud when it had one. The primordia which arose from neighbouring parts of the stem apex were often displaced towards the part to which the paste had been applied.

4. The subsequent phyllotaxis was often permanently changed, and in the *Epilobium* apices, which were at first decussate, it sometimes became spiral immediately after the paste was applied. This change can be explained as a consequence of the displacements of the primordia which developed immediately after the applications of the paste, on the basis of the theory, which we have supported previously (1931, 1933, 1935), that each new primordium arises in the first space which becomes available for it, through the growth of the stem apex, above and between the primordia which are already present.

5. The results are discussed and compared with the results of treating seeds or seedlings with X-rays, which is known to *diminish* the formation of auxin in one species at least (Skoog, 1935).

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THE DEVELOPMENT OF ROOT HAIRS BY *ELODEA CANADENSIS*

By R. G. H. CORMACK
Department of Botany, Toronto

(With 7 figures in the text)

It is characteristic of a number of water plants that they produce roots which are hairless in water but develop hairs when they penetrate the mud. Although this phenomenon has given rise to much speculation in the past no satisfactory reason has as yet been advanced to explain the hairless condition of the roots in water. In the present investigation an experimental and microchemical attack on this problem has been undertaken.

Elodea canadensis was chosen as the experimental material not only because it has been studied to some extent in the past but because it is of common occurrence and produces abundant roots under normal conditions.

Preliminary studies established the fact that the roots are perfectly hairless in water but that once they penetrate the mud hairs are abundantly produced. Figs. 1–3 represent strips of epidermis removed from various roots after warming with dilute ammonia, and illustrate the occurrence of special preformed hair-producing cells scattered haphazardly over the surface of the root. They are shorter than the neighbouring epidermal cells, and are distinguishable also by the greater density of their protoplasm. Fig. 1, from the meristematic region of a root growing in the soil, illustrates clearly the difference between the two types of cell. Fig. 2 is from the mature region of the same root, the circles representing the bases of root hairs. Fig. 3 is from the mature region of a root growing free in water. Such roots were always hairless, and their short cells are generally much longer than the haired cells of the soil roots. Though hairless they are clearly distinguishable from neighbouring long cells in the same region. In the older regions of the root they are not so easily recognized.

Such cells were first reported on *Elodea* roots by Schwarz (1883) and Sauvageau (1889). The former failed to produce root hairs by

growing the roots in contact with glass beads and ground glass, and thought that neither contact, chemical stimulation nor retardation of growth of the root was responsible for their production in the soil. Kroemer (1903) and Leavitt (1904) described similar cells in many other water plants. Miss Snow (1905) confirmed Schwarz's observations as to the production of root hairs in mud and their absence in water, but concluded that retardation due to the soil particles was the principal factor. Wilson (1936) was of the opinion that the production of root hairs in *Elodea* was not affected by light but was bound up with a stimulus which came into play when the root penetrated a substratum. The present writer (Cormack, 1935) concluded from work on a number of land plants that the production of root hairs depended upon a gradual change from pectic acid to calcium pectate in the primary wall of the elongating epidermal cell. Vertical elongation thus having been arrested while the cell was still actively vacuolating, the wall was pushed out at its softest point to form a root hair. Slightly alkaline calcium salt solutions were found to facilitate the reaction and thus to increase root-hair development.

The first experiments of the present investigation were designed to determine whether the same conclusions were valid in respect to *Elodea*. However, a long series of solutions which had been shown to produce root hairs in the land plants previously investigated utterly failed when applied to *Elodea* roots under similar conditions. It was thus shown that the failure to produce hairs in water was not due to a lack of available calcium for cell-wall formation.

At that time Wilson's paper had not been published and the next series of experiments was designed to show whether the presence of light was an agent in preventing hair formation in water roots. It was clear from casual inspection that these roots reacted differently to light from those of the land plants described in the writer's former paper. *Elodea* roots in water for example were always well supplied with chlorophyll.

Small plants of *Elodea* each bearing a root about 1 cm. in length were selected. The root was placed through a very narrow hole in a cork leading into a cylindrical glass vial filled with tap water. The vial measured 7·5 × 2·5 cm. and was covered by several layers of black paper. The plant was securely held in place by rubber bands and the vial was then placed on one side, on the bottom of a black glass jar filled with tap water. The roots were allowed to grow in the darkness for 3 or 4 days and at the end of that time they measured 3–5 cm. in length. On removal from the vial they were invariably

found to be covered by long hairs. In most cases, hairs had developed within a few millimetres of the point where the root entered the vial. In the most densely haired regions they measured 2–3 mm. in length. They were produced only by the short cells. Fig. 4 is from the mature region of a typical root grown in the dark and resembles very closely the epidermis of the root grown in the soil as illustrated in Fig. 2.

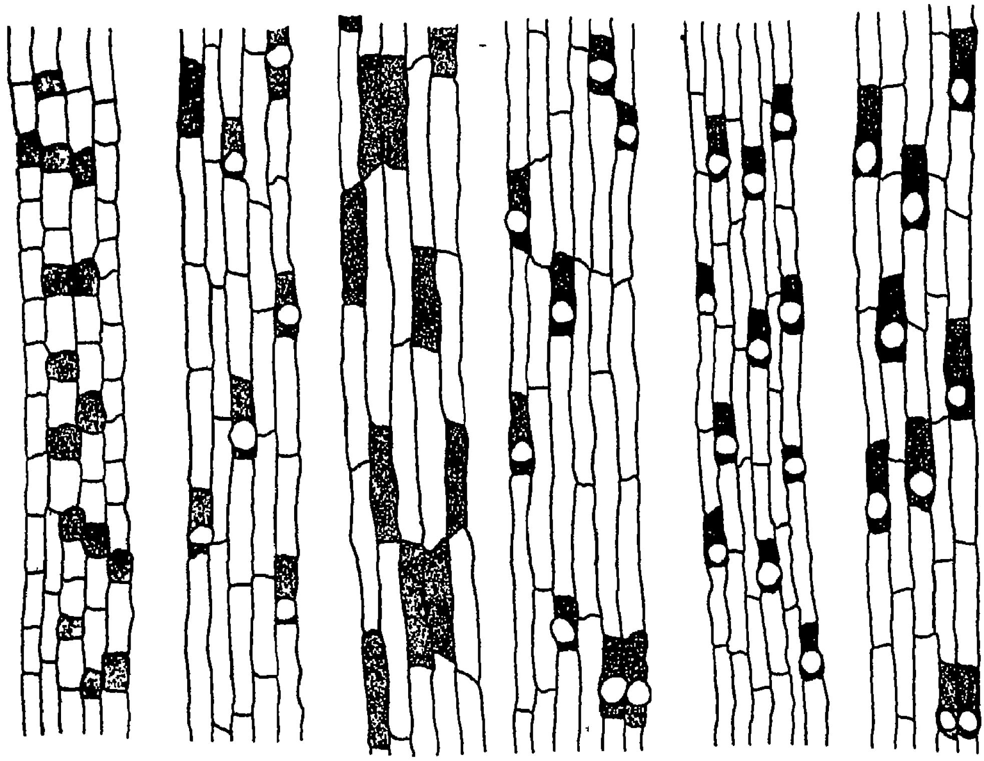
In the same way roots were grown in a number of slightly alkaline calcium salt solutions. In general the effect was greatly to stimulate cell division, more rows of cells being produced, and to increase the length and number of the hairs. Fig. 5 is from the mature region of a root developed in a solution containing 200 c.c. of saturated calcium sulphate solution and 0·25 c.c. of 1 per cent sodium hydroxide per litre tap water. Many of the hairs measured 5 mm. in length. These were the longest observed in this work. Hairs were never produced by the long cells, even in the most densely haired regions. Microchemical tests on all roots grown in the dark showed that the epidermal cell walls and the walls of the hairs themselves consisted of an inner layer of cellulose and an outer layer of calcium pectate.

The necessity of keeping the roots completely in the dark was clearly demonstrated by several experiments. One method was to allow a short root to grow into a slanting black coloured glass tube in a glass jar filled with tap water and exposed to the light. Although the roots attained a length of several centimetres, hairs were never developed. In another experiment a short root was allowed to grow into a long piece of black rubber tubing spirally coiled at the bottom of a cylindrical black coloured jar containing tap water. Although this root attained a length of 14 cm., only a few scattered short hairs were observed towards the extreme tip.

Since the publication of Wilson's paper in 1936 all the experiments were repeated with exactly the same results. His failure to produce hairs on roots growing in water in the dark must be attributed to the fact that some light entered his dark chamber. When light is completely excluded hairs are produced exactly as in other roots.

An investigation of the mechanism that prevents root-hair formation in light was next undertaken. A microscopic study of thin transverse sections of hairless roots brought to light two peculiarities. The first was the presence of chloroplastids in all the epidermal and most of the cortical cells. The second pertained to the outer wall of the epidermal cells. When treated with chlorzinc iodine, these walls showed outside the blue cellulose layer a thin but clearly marked

and continuous layer of brown stained material. This outer layer was also found capable of staining with Sudan III, and is quite clearly a cuticle. In roots grown in the dark whether in water or in mud both chlorophyll and cuticle were always absent. The epidermal cell



1 2 3 4 5 6

Figs. 1–3. Strips of epidermis from soil and water roots.

Fig. 1. Meristematic region of a root growing in the mud, showing the occurrence of both long and short cells.

Fig. 2. Mature region of the same root; the circles represent bases of hairs.

Fig. 3. Mature region of a root growing in the water. × 80.

Figs. 4–6. The production of hairs on *Elodea* roots without soil penetration.

Fig. 4. Mature region of a root developed in tap water in the dark.

Fig. 5. Mature region of a root developed in a slightly alkaline calcium salt solution in the dark.

Fig. 6. Mature region of a root developed in the light during treatment with ethylene gas. × 80.

walls in these roots consisted of a primary layer of pectic acid, changing under proper conditions to calcium pectate, and a secondary layer of cellulose. Chloroplastids in the roots of various water plants have been noted by Arber (1920), Kroemer (1903) and Noack (1921), and a cuticle on the epidermal cell walls of such roots has been observed by Nikolais, cited by Kroemer (1903), and Lee & Priestley (1924). No one, however, so far as the writer is aware, has suggested

any causal relationship between these two characteristics or any connexion between them and root-hair formation. The view as to the formation of cuticle as expressed by Lee & Priestley seems most reasonable in the present state of our knowledge. They believe that it is developed by the migration of fatty acids from the protoplasts of both external and internal tissues to the surface of the epidermis where they undergo condensation and oxidation. On this basis the production of cuticle in water roots in the presence of chlorophyll and its non-production where chlorophyll is absent is explainable on the ground that the oxygen produced by photosynthesis is necessary for the oxidation of the fats. Moreover cuticle is a tough non-plastic substance and once it has been formed its presence would be sufficient to prevent the lateral extension of the cell wall in the form of a hair.

The hypothesis to be tested is then as follows. In the light the roots produce chlorophyll and photosynthesis takes place, with the result that fatty acids migrating to the surface are oxidized and produce a cuticle. In darkness on the other hand root hairs may be produced in typical fashion, since the oxygen for the cuticle formation is not available.

To determine whether light in producing a cuticle and preventing root-hair formation acts through the medium of the chlorophyll, means were sought of preventing the formation of chlorophyll while supplying the roots with an abundance of light. In this connexion several papers by Crocker *et al.* (1931, 1935) were found most useful. These writers state that ethylene gas is effective in decolourizing green plant tissue. The idea suggested itself that this property of ethylene might be made use of to decolourize the roots of *Elodea* growing in light.

In order to expose the root to the gas without injuring the whole plant a special piece of apparatus was designed. As illustrated in Fig. 7, it consists of an outer glass cylinder *A*, 12 in. long and 1 in. in diameter and an inner glass tube *B*, ¼ in. in diameter, passing through rubber stoppers which close each end of *A*. A small hole was blown through one side of this inner tube and covered by a short piece of rubber tubing *C*. By means of a hot needle a narrow slanting hole was made in *C*, just above the aperture in the glass tube *B*. Inlet and outlet tubes were provided for changing the liquid in the outer cylinder *A*. In setting up the apparatus a small *Elodea* plant with a root about 1 cm. long was chosen and the root inserted through the holes in *B* and *C*, in such a way that it could be treated separately from the rest of the plant, which was kept submerged in

tap water in the outer cylinder. The root in the inner tube was also kept in fresh tap water except during treatment with ethylene gas. The treatment was carried out as follows. The tap water in tube *B* was drained out and the tube connected by means of rubber tubing to a large aspirator bottle containing the gas. The gas was first bubbled through a long column of tap water to ensure that it contained sufficient moisture and thus prevent the root from becoming desiccated. Gas was allowed to flow past the root for about a minute and then shut off. Both ends of the inner tube were then closed and the gas allowed to remain in the tube for a certain period of time. After treatment the tube was again filled with tap water. Pure ethylene was prepared by gently heating ethylene chloride in alcoholic potassium hydroxide.

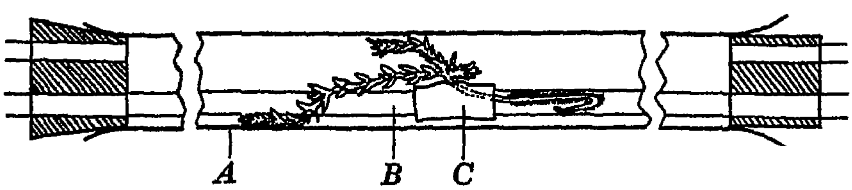


Fig. 7. Apparatus used in treatment of roots of *Elodea* with ethylene gas.

A number of experiments were performed before the proper treatment was discovered. If the supply of ethylene was insufficient the root remained green and hairless, while an excess of gas resulted in cessation of growth. The best results were obtained when the root was exposed for 2 min. periods, six times a day to a mixture of 1 l. of pure ethylene to 18 l. of air. The following description of a typical experiment with this treatment will illustrate its effect. At the beginning the root was green in colour and measured 4 cm. in length. At the end of 4 days' treatment as described above, it had attained a length of 7·5 cm., and was observed to be pure white in colour. Hairs were present over the whole surface of the portion developed during the experiment. These hairs measured 1–2 mm. in length and were conspicuous to the naked eye. Fig. 6 represents a piece of epidermis from this root. It compares almost exactly in appearance with the root developed in mud illustrated in Fig. 2, and the root developed in water in the dark illustrated in Fig. 4. Transverse sections through this root showed no chlorophyll, and no cuticle. In this respect they corresponded exactly to transverse sections obtained from soil roots and roots developed in water in the dark. Microchemical tests also showed that the epidermal cell walls and the walls of the hairs consisted of an outer layer of calcium pectate and an inner layer of cellulose.

Summary

1. *Elodea canadensis* belongs to a group of water plants whose soil roots produce root hairs while their water roots, under normal conditions, have none. In the epidermis of both types of root, potential hair-producing cells are clearly distinguishable.

2. When roots were grown in water in the dark, they were found invariably to produce hairs as abundantly as if they had been in soil.

3. Roots developed in water and in light are green and possess a cuticle. Those growing in darkness, whether in water or soil, have neither of these peculiarities.

4. When chlorophyll production is inhibited by means of ethylene gas without stopping growth of the root, a cuticle is not formed, and hairs are plentifully produced even in light.

5. The action of light in producing a cuticle and inhibiting root-hair growth, thus seems to be dependent on the presence of chlorophyll.

6. The hypothesis expressed is that the tough cuticle prevents root-hair formation, and for the production of this cuticle under water the oxygen set free by photosynthesis in the cells of the root is necessary.

I wish to thank Prof. H. B. Sifton not only for advice and assistance but also for his unfailing encouragement and enthusiasm throughout the investigation.

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THE FLORAL MORPHOLOGY OF *SPARTINA TOWNSENDII*

By WINIFRED M. CURTIS

(With 4 figures in the text)

Spartina Townsendii, a grass inhabiting tidal mud, was first recorded from the Southampton salt marshes in 1870 and in 1880 was recognized by H. & J. Groves (1880) as a distinct species. It is a rapid colonizer of soft muds and saltings, and is being used in the reclamation of muddy foreshores in Essex, Holland and other parts of the world. Some of these experiments are described by Oliver (1929) in a publication of the Ministry of Agriculture. Stapf (1926), who investigated and redescribed the plant, suggested that it might have arisen as a natural hybrid between *S. stricta* and *S. alterniflora*—an hypothesis which has since been confirmed by the cytological work of Huskins (1930). Huskins finds that *S. Townsendii* is "an allopolyploid derived from the doubling of the chromosome number in the original hybrid plant". This would account for the fact that for many years no segregates were found, though it is of interest to note that from plants introduced into New Zealand, Allan (1930) reports plants of *S. stricta* which are possibly segregates. Recently the status of *S. Townsendii* has been discussed by Senay (1934) and by Saint-Yves (1932). The latter in his *Monographia Spartinarum*, from a study of vegetative characters, places *S. Townsendii* as *S. stricta* sub. var. *pilosa*. The anatomy of *S. Townsendii* has been described by Sutherland & Eastwood (1916). An investigation of the floral morphology was made since it seemed to be of interest to examine the fertilization of this true breeding plant of apparent hybrid origin.

The inflorescence of *S. Townsendii* is a panicle consisting of three to eight spikes which carry sessile flattened spikelets arranged alternately in two rows. The spikelets are occasionally two-flowered, usually one-flowered when each consists of two empty glumes, one flowering glume and one palea. Lodicules are absent. There are three stamens. The number of styles is somewhat variable. Most commonly there are three of which two are fused for a short distance at the base, the stigmas being large and well developed. The third, anterior, style

is free, the stigma is sometimes smaller and does not always emerge from the glumes. In other specimens three separate styles are found, or two separate ones, or two fused for a short distance at the base. The specimens of the parent species *S. stricta* and *S. alterniflora* examined showed two free styles. Three styles, well known in some of the Bambuscae, are found occasionally in other species. Salisbury (1936) reports their occurrence in *Briza media*; here also the anterior style is less well developed.

Flowering takes place from July until November with a maximum in September. The flower is protogynous and is wind pollinated.

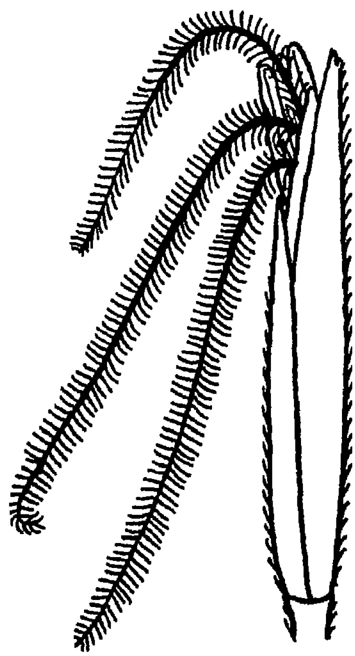

Fig. 1. Flower of *S. Townsendii*, ×3, showing exceptional development of stigmas. The flower was covered when mature to prevent pollination and collected 7 days later.

Observations on the maturation and duration of the flower made at Christchurch Harbour in September 1929 show that when the first spikes of the inflorescence emerge from the sheath the stigmas of the flowers are protruded, increase in length and persist for 3 or 4 days. The stamens emerge on the fifth day. When pollination is prevented the female stage persists for a longer time during which the stigmas continue to grow.

Anthesis takes place from about 8 a.m. After the anthers are visible they become completely free from the glumes in 1½ hours after which time the filaments continue to elongate rapidly, becoming as long as the anthers after a further interval of 60–80 min. Frequently the developing stamens push out the top portions of the

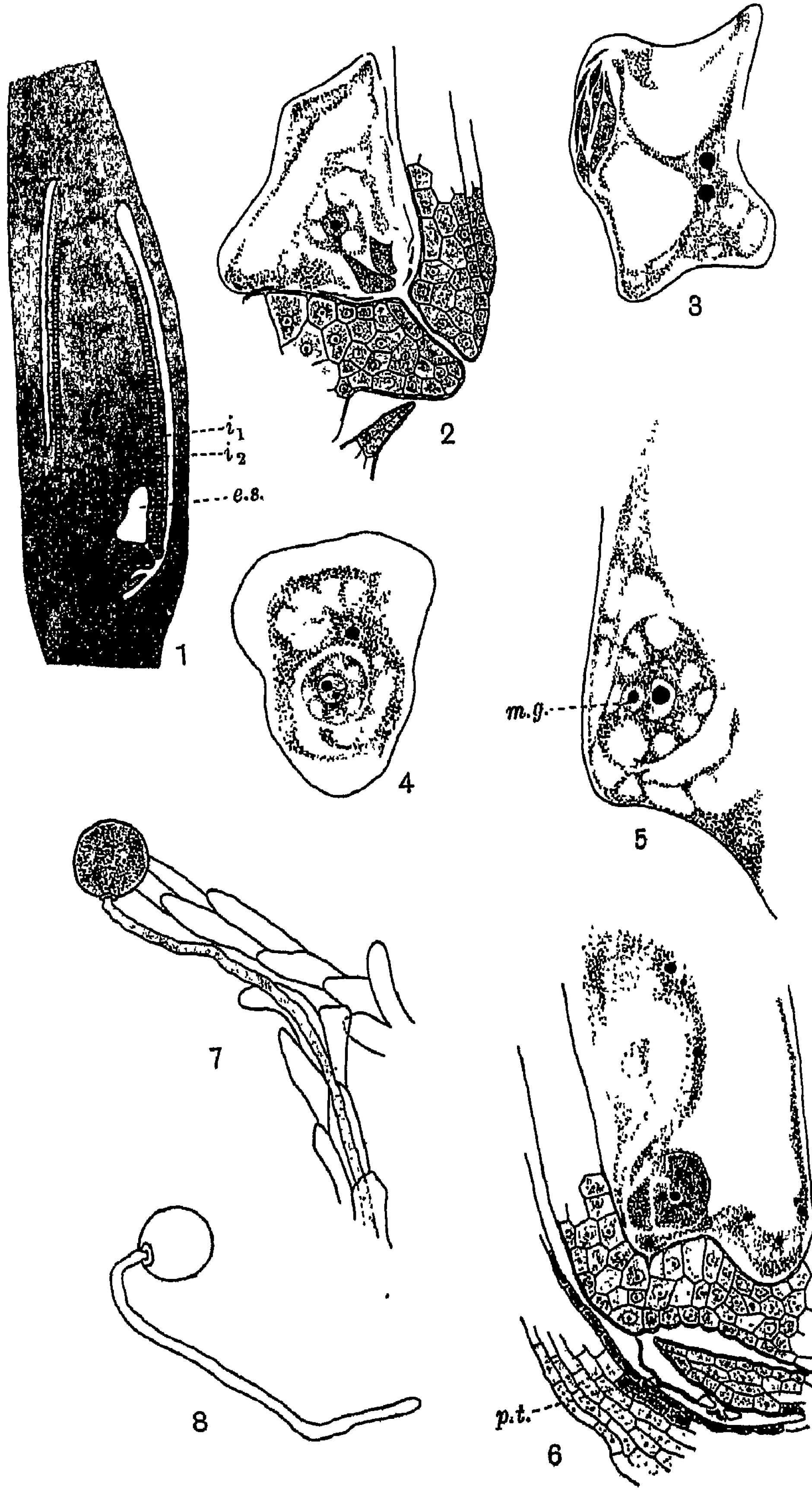

Fig. 2.

withered stigmas. The anthers dehisce in succession, the first may show a split at the apex before it is free from the glumes. This split extends rapidly down the length of the anther; later the edges curl back towards the connective. At this stage pollen is shed in clouds on shaking the plant. The anther and filament shrivel 50–60 min. after dehiscence. Pollen is carried by the wind to the feathery stigmas. To test the wind carriage of pollen, glass slides with a drop of glycerine were exposed 15 ft. south-west and north-east of a *Spartina* patch 128 ft. wide, the wind being very slight and in a south-west direction. The slides were placed at the level of the inflorescences and were left at 8.20 a.m. for ½ hour. One pollen grain was found to have travelled against the main direction of the wind and five with it. The grains were turgid and capable of germination.

The pollen germinates readily on the stigmas and may be seen in material mounted in lactic phenol to which a little cotton blue has been added. After 1 hour pollen tubes having a length nine or ten times the diameter of the pollen grain are found. After longer intervals the tube passes down the barb of the stigma into the main axis. The pollen also germinates easily in a moist chamber—a method devised by Anthony & Harlan (1920) for barley pollen. Of 299 pollen grains shaken from a mature anther and placed on a dry cover-slip over a Ward tube containing a drop of water, 94 per cent germinated. The maximum growth of the pollen in culture is shown in the diagram made 4 hours after sowing. There was no indication of any appreciable percentage of abortive pollen in this material collected in October 1928, but material collected in November after heavy storms showed all the pollen grains empty and shrivelled.

The unilocular ovary contains one basal ovule, the orientation being between that of an anatropous and campylotropous ovule. The nucellus is enclosed by two integuments, the inner cells of the inner integument are transversely elongated, the inner wall being cuticularized as is also the outer wall of the outer integument. The

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Fig. 2. Diagrams made with camera lucida. Magnifications: 1, ×38; 5, ×380; others, ×180. 1, longitudinal section of ovary, slightly oblique at free end: i_1 inner integument; i_2 outer integument; *e.s.* embryo sac. 2, longitudinal section mature embryo sac showing egg nucleus and synergids. 3, longitudinal section mature embryo sac showing polar nuclei and antipodals. 4, transverse section embryo sac 27 hours after pollination showing egg nucleus with two nucleoli. 5, longitudinal embryo sac 28 hours after pollination: *m.g.* male gamete. 6, longitudinal section embryo sac 48 hours after pollination showing proembryo and endosperm development: *p.t.* remains of pollen tube. 7, pollen grain germinating on the stigma. 8, pollen grain grown in moist chamber, after 4 hours.

mature embryo sac contains one egg cell, two polar nuclei, two synergids and a group of about fourteen antipodal cells. A group of antipodal cells has frequently been recorded in monocotyledons, as in *Triglochin maritimum* described by Hill (1900). The embryo sac may also be compared with that of *Avena*, described by Cannon (1900), and with that of the sugar cane investigated by Artschwager *et al.* (1929).

In an attempt to follow the growth of the pollen tube through the style to the ovary, nearly mature inflorescences were selected, covered with manilla bags, and the stigmas pollinated when they were receptive. The experiments were made in the mornings from 8 to 10 a.m. and the pollen was taken from anthers as they dehisced. Ovaries from the pollinated flowers were collected at intervals of 1 hour for 60 hours and at intervals of 12 hours until 10 days after

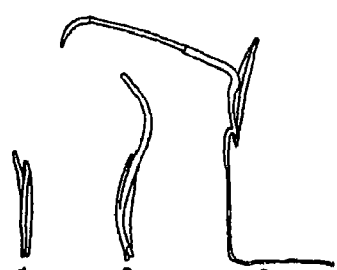

Fig. 3. Germination of *S. Townsendii*. Diagrams × $\frac{2}{3}$. 1, after 6 days; 2, 11 days; 3, 27 days.

pollination. The ovaries were dipped into acetic alcohol and transferred to chromo-acetic solution. Sections were stained in iron alum-haematoxylin or in Delafield's haematoxylin.

The pollen was seen to germinate on the stigmas but the growth of the pollen tube to the ovary has not been followed completely. There is, however, evidence of fertilization, which would appear to be normal. In a preparation made 28 hours after pollination a male gamete is seen in the egg cell, in one made 27 hours after pollination there are two nucleoli inside the egg nucleus. In two other preparations the apparent remains of pollen tubes are found near the micropyle between the outer integument of the ovule and the ovary wall and proembryos are in the positions previously occupied by the egg cells. In all these, endosperm formation by free cell division is beginning and the antipodals are degenerating. Seven or eight days after pollination the embryo is seen to be placed by means of a long suspensor into the developing endosperm.

Counts of 2000 withered flowers collected from Poole Harbour in

October 1928 showed that 40 per cent contained well-developed fruits. The fruits when shed germinate freely, numerous seedlings apparently a few weeks old having been seen in the drift towards the end of October. Eighty of the fruits collected in October were placed in Petrie dishes on moist sand when 77·5 per cent germinated during the period October–April, after which the cultures became unhealthy. The graph shows that germination is continuous. In April 1929, seventy-five Dutch fruits collected in the previous autumn gave

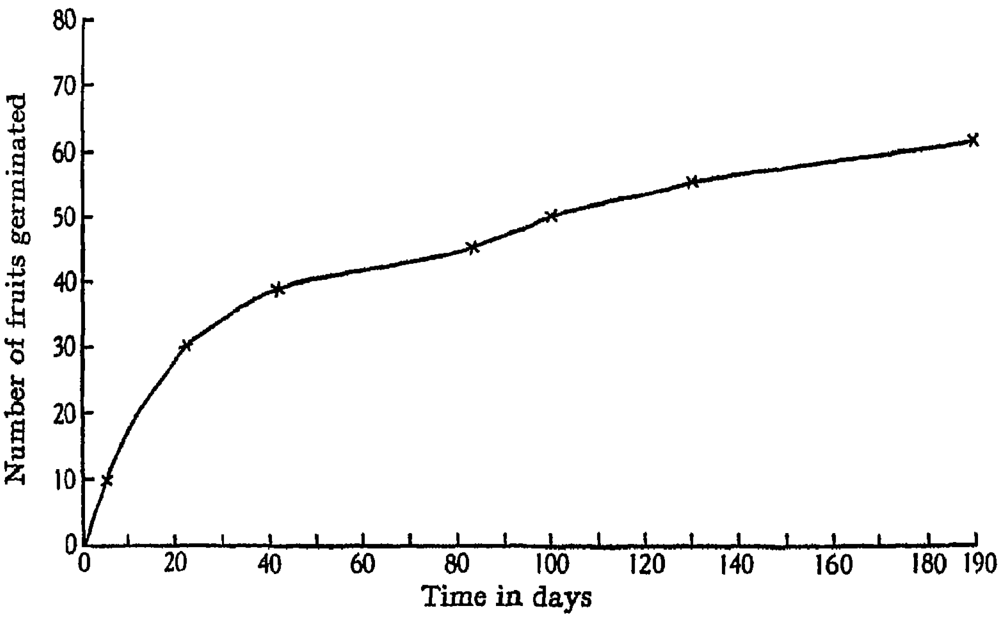

Fig. 4. Graph showing the germination of sixty-two fruits of *S. Townsendii* from eighty planted in October 1928.

64 per cent germination within 6 weeks. Of 160 English fruits collected in November 1929, 65 per cent had germinated by February 1930. An interesting feature is that in all instances the plumule emerged from the pericarp some days before the radicle. The seedlings, however, established themselves and plants from them were in cultivation for some years. This method of germination affords an interesting comparison with the "false germination" reported in several grasses as an occasional phenomenon during seed trials, such "false germination" usually being counted as failures. Recently, germination tests have been conducted by Nelson & Munro (1934) to find the conditions of storage necessary if the fruits are to remain viable when sent for the colonization of new habitats.

This investigation was started at University College, London, at the suggestion of Prof. F. W. Oliver to whom I am very greatly

indebted for advice and encouragement. Prof. Montagu Drummond kindly allowed me to continue the work in the Botanical Laboratories of the Victoria University of Manchester. I should like also to express my thanks to the Director of the Royal Botanic Gardens, Kew, for allowing me to work in the Jodrell Laboratory, and to Dr C. R. Metcalfe and Dr W. B. Turrill for advice during the preparation of the paper.

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A REVIEW OF RECENT WORK ON THE NITROGEN METABOLISM OF PLANTS, PART I

By H. S. McKEE
Department of Botany, Oxford

CONTENTS

INTRODUCTION

The purpose of this review is to give an account of the work done recently on the nitrogen metabolism of plants and to attempt an assessment of our knowledge of its problems. The article by M. E. Robinson in the 1929 issue of this *Journal* is taken as a starting point, though I propose to cover a rather wider field and reference is made to a number of papers published before 1929 but not mentioned by Robinson.

In reviewing so wide a field of work a number of difficulties arise. Much work, though published, remains inaccessible in the numerous *Journals* which are not available in the majority of libraries, and especially in the reports of American and Russian research stations. The results of different workers are often difficult to compare, owing to variations in technique and the manner in which the results are reported. It is, for instance, often impossible to assess the value of work the results of which are given per unit of dry weight only. Again, as plant physiologists necessarily work with complex mixtures

of substances in their extracts, the reliability of most methods is not certain, and unrealized inaccuracies in the methods used may lead to contradictory results by different workers.

Perhaps the most striking characteristic of our present knowledge of nitrogen metabolism in the plant is the extent to which it is confined to down-grade processes; this is a general feature of physiology, for while catabolic processes proceed freely *in vitro* it is not yet possible to reproduce artificially the conditions necessary for synthesis and co-ordination.

No comparative account of the nitrogen metabolism of plants, on the lines of Needham's (1929) brilliant paper on nitrogen catabolism in the different animal phyla, is at present possible. Among autotrophic plants only the flowering plants have been extensively studied, though Haas & Hill (1931) and Haas *et al.* (1935) have begun work on the higher marine algae.

THE ABSORPTION AND ASSIMILATION OF NITROGEN BY PLANTS

(a) *Inorganic compounds*

M. E. Robinson, in her review, has given an account of the earlier work of Pryanishnikov on the assimilation of ammonium salts by plants, while rather later (1931) he summed up his view of the matter by stating in a paper written with Ivanova: "Normal plants always assimilate ammonia more energetically than nitrates." Much work has since been done on the relative availability to plants of nitrogen supplied as ammonium salts and as nitrates. The results have been well summarized by Pardo (1935), but, as he points out, it is difficult to draw any definite conclusions from the mass of data available. There are several reasons for this. Most of the work has been done as agricultural research and has been concerned with variables, such as fresh weight, dry weight, fruit production, tuber formation and chlorophyll content, which cannot readily be evaluated in metabolic terms; secondary factors have a marked effect on the uptake of both forms of nitrogen; the behaviour of different plants, of different parts of the same plant, and of the same plant at different stages of its life history, varies widely; and the presence of bacteria in all soil and in most sand and water cultures makes it uncertain what proportion of the nitrogen supplied as ammonium or as nitrate actually reaches the plant in those forms. It has even been shown (Dhar *et al.* 1933; Corbet, 1934) that the sequence ammonia→nitrite→nitrate can

occur in soil in the total absence of bacteria, the first reaction being photochemical and the second catalysed by the hydrogen ion.

A brief description of the results of some recent work on the effects of secondary factors on the absorption of ammonium salts and nitrates may serve to give an idea of the complexity of the problems involved.

Acidity. Pryanishnikov (1929) found that sugar beet takes up ammonium better than nitrate at *p*H 7, but that the position is reversed at *p*H 5. This effect is complicated by that of calcium, for even at *p*H 7 nitrate is taken up better than ammonium if the supply of calcium is reduced. Richmond & Pearsall (1931) found that above *p*H 5·5 the rate of absorption of ammonium by discs of potato tuber increased with *p*H, while that of nitrates decreased. They found also that plants of *Eriophorum* and wheat showed minimum nitrate absorption at *p*H 4·1 and at *p*H 7, and higher values at intermediate acidities. *Eriophorum*, in contrast to wheat, absorbed more ammonium as the *p*H fell, while in all circumstances it absorbed only one-third to one-fifth as much nitrogen per unit dry weight as the wheat. These peculiarities may possibly be correlated with the acid tolerance of *Eriophorum*, but until further information is available about the behaviour of plants growing under similar ecological conditions, it is impossible to generalize. Arrington & Shive (1935) report, in contrast to the other results mentioned, that at *p*H 4 the ratio of absorption of ammonium to that of nitrate was 4·7, while at *p*H 7 it was 1·2.

Stage of developement. The work of Stahl & Shive (1933*a*, *b*) has shown that the absolute rates at which a plant absorbs the two forms of nitrogen may vary considerably during its life history. For oats the rate of absorption of nitrates is minimal in the young seedling, rises to a maximum at the time of flowering, and then declines rapidly again, whereas ammonium is most rapidly absorbed in the early stages and thereafter the rate declines steadily to maturity. Thus for the seedling ammonium is a better nitrogen source than nitrate, but not at the time of flowering. With buckwheat (*Fagopyrum esculentum*) the rate of ammonium absorption is high at first, rising to an early maximum, and then slowly falls away. As with oats, there is a maximum rate of nitrate absorption at the time of flowering, and later a steady decline. A connexion of nitrate absorption with flowering has also been noted by other workers. Campbell (1924) has shown that a large number of annual and biennial plants absorb nitrates at a high rate in the earlier stages of developement, and store

considerable amounts in their aerial parts. These nitrates disappear at the time of flowering, and the nitrate content of the leaves remains thereafter very low. Culpepper & Caldwell (1932) found conversely that the leaves of rhubarb plants which were not allowed to flower accumulated nitrate steadily until they died. There are unfortunately no corresponding data for flowering rhubarb plants. Penston (1935) has summarized the results of several Rumanian workers, who found in the later stages of developement a considerable return of mineral elements, including nitrogen, from plant to soil. According to Burd (1919) the absolute nitrogen and potassium contents of barley plants decrease by over 30 per cent from the eighth to the eleventh week of growth, and later increase again. Pryanishnikov and Ivanova (1931) have also found that the roots of plants with deficient carbohydrate supplies may excrete ammonia.

Pearsall & Billimoria (1936) have pointed out another way in which nitrogen can be lost by the plant. The nitrite formed in the reduction of nitrate may be expected to react, in acid saps, with amino-acids to form gaseous nitrogen:

$$R.NH_2+HNO_2=R.OH+N_2+H_2O.$$

It was shown that *Chlorella vulgaris* and detached daffodil leaves lose a large part of the nitrogen taken up from nitrate media and that no such losses occurred with ammonium media.

Carbohydrate supply. The uptake of ammonium, but not that of nitrate, is affected by the amount of available carbohydrate in the plant. Free ammonia is a cell poison, and if not converted to another form is dangerous to the plant. For this conversion non-nitrogenous compounds derived from carbohydrates are required, and in carbohydrate shortage ammonium is clearly a less suitable form of nitrogen supply for the plant than nitrate, which can be stored as such with no ill-effects, though it cannot in these circumstances be used in synthesis. This accumulation of nitrates may go on to a remarkable extent without harmful effects. Nightingale *et al.* (1930) grew soy beans with and without added nitrate, using two lengths of day. With the short day there was no difference in appearance between the two sets, but the plants receiving nitrate contained three times as much total nitrogen, 56 per cent. of which the authors state was nitrate, as the others. The nitrate determinations were made by the method of Sessions & Shive (1928) which is reliable in the presence of most of the forms of organic nitrogen likely to be present in plants, with the probable exception of glutamine. In the plants grown with

a long day, those receiving extra nitrogen were much bigger than the others, the nitrates which had piled up unchanged in the "short-day" plants, owing to the shortage of carbohydrates, having been used here in greater growth. A very high supply of nitrate may also lead to its accumulation in plants with a normal carbohydrate supply. Chibnall & Miller (1931) found that *Lolium perenne* grown on a sewage farm contained 12·7 per cent of its total nitrogen as nitrate. These observations show that if the amount of nitrate taken up is greater than that synthesized to complex products, the excess can be stored as such without any harm to the plant. A high nitrate supply can also, as Gassner & Franke (1934) have found with wheat seedlings, lead to an increase in amides. Some plants, especially members of the Chenopodiaceae (Dittrich, 1930) are known normally to store large quantities of nitrates and to be characterized by a low content of the enzyme which catalyses their reduction. This metabolic peculiarity may be correlated with the characteristic habitats of many members of this family in situations where the supply of organic matter, and thus of nitrates produced by micro-organisms, is high. In such circumstances the uptake of nitrate is likely to be high, and a plant with a high reductase content would be at a selective disadvantage, as unduly large quantities of ammonia would be formed in its tissues.

(*b*) *Organic compounds*

Nitrate and ammonium have till recently been regarded as the only possible nitrogen sources for plants growing naturally, but the work of Virtanen and his collaborators at Helsinki has shown that this is not so. They found that the bacterial nodules on the roots of leguminous plants excrete considerable amounts of organic nitrogen into the surrounding medium, especially if it is solid. Virtanen & Laine (1935) have shown that about half of this nitrogen is excreted as aspartic acid and the rest mainly as lysine. The absence of a number of amino-acids invariably found in protein decomposition indicates that the compounds set free from the infected plants are not due to the decay of the nodules or of the roots. It has also been shown (Virtanen *et al.* 1933; Thornton & Nicol, 1934) that these compounds can be used by non-leguminous plants growing in association with the legume. Red and white clover have also been shown by Virtanen & von Hausen (1931) to grow much better in sterile solution if supplied with amino-acids than with ammonium salts or nitrates. The amino-acids are taken up unchanged, which

supports Virtanen's theory that the legumes receive nitrogen in this form from their root nodules. Virtanen & Saastamoinen (1936) have also investigated the behaviour of the alder, a non-leguminous plant with root nodules, from this viewpoint. They find that over a wide range of acidities seedlings grow better when infected with nodule bacteria than when supplied with nitrates or ammonium salts. It was also found that a pine seedling could get its nitrogen from an alder grown in the same pot. The alder has also been studied in this connexion by Roberg (1934) who could not detect any excretion of nitrogen into the surrounding medium, a result probably to be attributed to the fact that he used water cultures, for the Finnish workers have found that legumes excrete fifty times as much in sand cultures as in water.

Later work (Virtanen, 1936) has shown that the extent of excretion is greatly affected by the absorptive power of the surrounding medium, and that the presence of other plants causes a marked stimulation. In some cases the associated plants may get much more of the nitrogen fixed in the nodules than the legume itself.

From these results it seems possible, especially in pasture, that an appreciable amount of a plant's nitrogen supply may be taken up in organic form, as there must be present in the soil, besides amino-acids set free from root nodules, those formed in the decomposition of dead organic matter. The subject is reviewed at length by Nicol (1934), who also considers a number of other problems connected with nitrogen transformations in the soil.

(c) *Gaseous nitrogen*

The old controversy about the ability of the higher plants to assimilate gaseous nitrogen has once more been revived, though with the usual inconclusive results. Vita (1932*a*, *b*) has claimed that leguminous seedlings grown in sterile conditions in the presence of carbon monoxide or of certain alkaloids can assimilate free nitrogen. This work has been repeated by Smyth & Wilson (1935), who found an apparent increase in the nitrogen content of germinating pea seedlings both in the presence of Vita's stimulants and in distilled water. They attribute the results to the inadequacy of the Kjeldahl method of nitrogen estimation and note that the nitrogen content of the initial seed samples was very variable, as was also found by Orcutt *et al.* (1934) and by Girtschanoff (1935). The latter was unable to confirm Vita's results, which he attributes to inadequate controls,

the variability of the material, the unreliability of the Kjeldahl method, and to an uptake of nitrogen by the seedlings from the cotton-wool used in the experiments. In the papers of Vita and Smyth & Wilson the results are expressed in terms of nitrogen content per unit dry weight, which suggests that the observed increases might be due to the loss of carbohydrate during germination; but Vita in later papers and Wilson in a personal communication have made it clear that the original dry weight of the seed sample was meant, so that there is an apparent absolute increase. Virtanen's work, discussed above, shows incidentally that if sterile leguminous seedlings do assimilate any gaseous nitrogen, it is not enough for them to make satisfactory growth.

The nitrogen relations of the liverwort *Cephaloziella byssacea* are discussed in papers by Griggs (1933) and by Griggs & Ready (1934). They found, while investigating the return of vegetation to an area in Alaska which had been covered with ash by an eruption of the volcano Katmai, that the only plants growing actually on the ash, which contained extremely little nitrogen, were leafy liverworts belonging to the Jungermanniaceae. In artificial cultures specimens increased in size several hundred times in nitrogen-free liquid media, adequate precautions being taken to prevent any gaseous nitrogen compounds from reaching the plants. There was no definite evidence that the liverworts fixed free nitrogen, as the cultures finally weakened and could be restored by the addition of one part of ammonia per million, and the test used for nitrates on the original medium would not have demonstrated such a concentration. The authors suggest that at Katmai the liverworts may be able to get enough nitrogen for their requirements, which it is clear must be remarkably small, from the ammonia in rain water, though it has been shown that the rain there contains much less ammonia than that of industrial regions.

The suggestion that willow cuttings can assimilate atmospheric nitrogen has been put forward by Hicks (1928), but Ludwig (1934) was unable to confirm this.

The formation of amino-acids and protein in the plant

Ammonia is probably to be regarded as the starting point in amino-acid syntheses; as Pryanishnikov puts it: "Ammonia is the alpha and omega of the transformations of nitrogenous substances in the plant." Its position may in some respects be compared with that of carbon dioxide in carbohydrate metabolism and the fact

noted that the most reduced form of nitrogen and the most oxidized form of carbon play similar metabolic roles. The oxides of nitrogen seem to play no part in metabolism, while the hydrocarbons are but rarely found.

If the plant absorbs its nitrogen as nitrate, this must first be reduced. Nitrite is presumably an intermediate step, and Eggleton (1935) has shown that nitrites increase in herbage which has been supplied with nitrogen either as nitrate or as ammonium salts. Mevius & Dikussar (1930) considered that nitrite itself was a very good nitrogen source for maize, since the nitrogen content of the plants rose more rapidly when they were supplied with nitrite than with either nitrate or ammonium. As, however, the nitrogen content was expressed as a percentage of the dry weight, it is possible that this observation was due to a reduction in carbohydrate production rather than to an increase in the uptake of nitrogen. Hydroxylamine is another possible intermediate between nitrate and ammonia and has been found, though mostly in oxime form, in *Poa pratensis* by Lemoigne *et al.* (1935).

The method of formation of amino-acids from ammonia and non-nitrogenous precursors is still obscure. It has often been thought that, as this synthesis occurs only in the plant, it may be a photochemical process dependent in some way on the chlorophyll complex. Nightingale & Schermerhorn (1928) and Nightingale & Robbins (1928) have, however, shown that it occurs in the dark in the roots of *Narcissus Tazzetta* and *Asparagus*. On the other hand, Dhar & Mukerjee (1934) have found that amino-acids can be synthesized photochemically *in vitro*, using titanium dioxide as a catalyst. They obtained arginine from glucose in the presence of potassium nitrate, and Eggleton (1935) has found that amino-acids are photosynthesized *in vitro* from nitrites and sugars. Fromageot & Desnuelle (1934) found that yeast only synthesizes amino-acids when actively fermenting and suggest that there is a connexion between the oxidative processes going on in fermentation and the reduction expressed in the equation:

$$CH_3.CO.COOH+NH_3+H_2=CH_3.CHNH_2.COOH+H_2O.$$

Both the hydrogen and the pyruvic acid would be products of fermentation. It is probable that the non-nitrogenous precursors of amino-acids are intermediate products in glycolysis, and their concentration must thus have an important effect on the regulation of amino-acid and protein metabolism in the plant. This point will be

further discussed in the sections on the regulation of protein breakdown and amides.

Our knowledge of the synthesis of protein from amino-acids is no more satisfactory than that of the earlier stage. The two hypotheses outlined by M. E. Robinson (1929), (1) that each amino-acid is formed separately and then condensed, and (2) that the proteins are formed by the polymerization of units formed from comparatively simple compounds, are both still tenable. The extensive work of Björkstén (1930) on the synthesis of protein by detached wheat leaves supplied with a large number of nitrogenous and other compounds by vacuum infiltration has, however, provided interesting data. He found that aliphatic amides, amino-acids, the lower amines, and the ammonium salts of organic acids were all good nitrogen sources for the synthesis of protein, while nitrates and the ammonium salts of inorganic acids are much less effective. This may indicate that the leaves do not normally synthesize protein direct from inorganic compounds, but from more complex precursors translocated from other parts of the plant, or merely that the duration (6 hours) of Björkstén's experiments was not long enough to allow the formation of protein from inorganic sources. Björkstén concludes that amino-acrylic acid, which he suggests may arise by the action of ammonia or amides on pyruvic acid, is the simplest nitrogenous precursor of protein. Similar experiments by Björkstén & Hinberg (1930) show that although wheat leaves do not split off any ammonia from acetamide and butyric amide, yet they can use these compounds for the synthesis of protein, whence it is concluded that the formation of ammonia is not an essential stage in protein formation.

A study of the proteins of the barley seed during its developement (Bishop, 1930) shows that during ripening the content of salt-soluble compounds decreases, with corresponding increases in the insoluble proteins, hordenin and glutelin. This is in general agreement with the results of various older workers with other ripening seeds. An increase of insoluble protein and a decrease of soluble compounds during ripening is found in the classic work of E. Schulze (1911) on *Pisum sativum*, and in that of Sobolevskaya & Turetskaya (1931) working with red currants. Even in the dry seed, however, there are appreciable amounts of soluble compounds. E. Schulze & Flechsig (1886) reported considerable amounts of amides in cereal seeds, especially rye, but some at least of their amides may actually have been glutamyl peptides, which, if they have a free amino group in

the γ position relative to the amide group, are estimated as amides by most methods (Melville, 1935). Bhagvat & Sreenivasaya (1935) have studied the peptides which are present in a number of Indian pulses and find that they are mostly simple compounds built up from a few amino-acid molecules. In underground storage organs the content of soluble nitrogen compounds is characteristically high. As much as 70 per cent of the total nitrogen may be soluble (Grüntuch, 1929; Rahn, 1932).

Bishop (1930) has found that in barley the relative contents of hordenin (soluble in 75 per cent alcohol), glutelin (alkali soluble), and salt-soluble compounds vary from one variety to another, and also that in mature grain of any one variety the relative content of salt-soluble protein falls as the total nitrogen of the grain rises, while the relative amounts of glutelin and hordenin increase.

The plant proteins

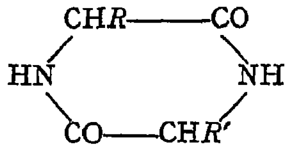
Much work continues to be done on the plant proteins, but the results are often such as to render generalization difficult. The comment of Kiesel *et al.* (1934) is very appropriate. "There remains much hard work to be done before we can overcome the great difficulties in preparing proteins from plant organs, so that we can accurately test their genetic relationships." There are two main causes of this state of affairs, the difficulty of obtaining the plant proteins in a pure form and the chemical difficulties of their characterization. At present the only criteria much used are the relative proportions in which the various amino-acids are formed on hydrolysis and various physical characteristics such as the isoelectric point. It is thus clear that only gross differences between proteins can be detected, the multitudinous possibilities of isomerism of any protein molecule escaping our present chemical methods.

Kiesel *et al.* (1934) have recently reprinted results from two earlier rather inaccessible papers, together with some new work. They were unable to establish significant differences in the seed proteins of selected varieties of *Phaseolus vulgaris*, *Pisum sativum* and wheat; characterizing the proteins by their nitrogen content, their content of various amino-acids and their isoelectric points. They did, however, find differences in the nitrogen distribution in the hydrolysis products of proteins from leaves and tubers of the potato, and in those from the leaf and root in sugar beet and from leaves of sugar beet and red beet. Kiesel & Kastrubin (1934) found marked differences in the

distribution of nitrogen among the various amino-acids in proteins extracted from ripening wheat seeds on different occasions from July to October. Leontyev (1934) reports that globulins from a number of cucurbitaceous seeds are identical, while Hirohata (1932), who examined the globulins from various species and varieties of eight cucurbit genera, found them all similar (except for *Momordica*), but there were slight differences in the contents of individual amino-acids. Csonka & Jones (1933) found differences, which they believed to be significant, in the contents of cystine, tryptophane and tyrosine (they varied respectively from 0·74 to 1·76, from 1·89 to 2·84 and from 3·94 to 4·55 as percentages of the total protein), in the proteins of ten varieties of *Soja maxima*. Blagoveshchenski (1924) found that the proteases of a number of plants hydrolysed their own proteins more readily than those of other plants, and Blagoveshchenski and Melamed (1934) showed that such differences could be qualitative as well as quantitative. N. N. Ivanov's results (1932), on the variability of the chemical composition of different varieties of the same species and of members of the same variety grown in widely separated places, indicate a considerable lack of fixity and show that the chemical characteristics of a species are as much affected by differences in heredity and in external conditions as are morphological features. The finding of Schenk & Kunstmann (1933), that during the day the relative quantities of the different amino-acids in the proteins of the blood serum of the same individual vary, presumably in relation to metabolic processes continuously building up and breaking down the proteins, further illustrates the difficulty of satisfactorily characterizing individual proteins, especially those derived from active tissues.

The nature of the linkages in the protein molecule is not yet definitely known, but there is new evidence which supports the idea that there are in the molecule, besides peptide linkages, others of a cyclic type, with nitrogen atoms in the ring. One objection to this theory was that no diketopiperazine derivative or similar cyclic anhydride had been hydrolysed by a proteolytic enzyme, but Shibata (1934) and Tazawa (1935) have shown that a number of such compounds, of the general formula below, and all having a free amino or carboxyl group, are hydrolysed by trypsin, pepsin and papain (Formula I). Waldschmidt-Leitz & Kofranyi (1935) have stated that they cannot confirm this work, but their detailed results have not yet been published. Blagoveshchenski & Sossiedov (1933) and Blagoveshchenski & Yurgenson (1935) have shown that flour enzymes

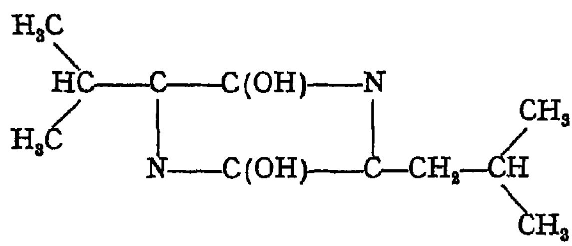
disaggregate cereal proteins without setting free amino groups, which indicates that the peptide linkage is not being attacked. Anhydrides have been isolated from the products of comparatively mild protein



......(I)

$R = R' = CH_2NH_2$ in diaminopropionic anhydride.
$R = H$, $R' = CH_2.CH_2.COOH$ in glycylglutaminic anhydride.
$R = R' = CH_2.COOH$ in aspartic anhydride.

breakdown, but it is not yet definitely established that they are not artefacts. Sadikov & Lindquist-Rysakova (1935) have isolated the following cyclic amino-acid anhydride from the products of hydrolysis of blood albumin (Formula II).



......(II)

It has long been thought probable, owing to the large amounts of ammonia and of aspartic and glutamic acids formed on the hydrolysis of plant proteins, that asparagine and glutamine exist as such in the protein molecule, but it is only recently that this has been demonstrated experimentally. Damodaran (1932) isolated asparagine from an enzymic digest of edestin from hemp seed, and Damodaran *et al.* (1932) similarly obtained glutamine from wheat gliadin.

The proteins of tissues other than seeds are still little known, but Miller (1935, 1936) has published chemical data on the leaf proteins of *Dactylis glomerata* and Kiesel *et al.* (1934) on those of the potato and sugar beet. Chibnall (1923, 1924, 1926) has extracted separately the proteins of the cell-sap and cytoplasm of a number of leaves. The chief difference between the two types of protein was the lower nitrogen content of that from the vacuole, which might conceivably have been due to contamination with carbohydrate.

Chibnall & Grover (1926) have found that, except in such acid plants as *Rumex acetosella* and *Vitis vinifera*, the proteins exist in the cell as anions. This confirms the suggestion of James & Penston (1933) that they may form potassium salts, which may be responsible in part for the observed similarities between the distribution of

protein and potassium. Pearsall & Ewing (1929) found that the acidity of the cell sap of radishes, turnips, and swedes decreased with increased nitrogen supply, and that the plants became more succulent, which they attribute to the increased water uptake and swelling of the proteins when they are further removed from their isoelectric points. Chapman (1931) disputes this conclusion, on the grounds that other investigators have found that the reaction of the cell sap has no effect on succulence and that he found that nitrogen starvation led to succulence in *Tradescantia fluminalis*.

THE CHEMISTRY OF PROTEIN BREAKDOWN

A vast mass of data has accumulated concerning the specificities of the protein-splitting enzymes, but it is difficult to give a generalized picture of the situation. Grassmann, who summarized the work on this subject in 1932, made three general statements.

(1) There is as yet no known proteolytic enzyme whose action is purely a disaggregation. Such an enzyme has, however, since been found (p. 43) by Blagoveshchenski and his co-workers.

(2) No enzyme is yet known which will split diketopiperazine derivatives. Through the work of Shibata and Tazawa (p. 43) this is no longer true.

(3) In enzymatic protein breakdown amino and carboxyl groups always appear in equivalent amounts. This statement still stands, except for enzymes splitting the CON linkage in prolyl peptides (Grassmann & Schneider, 1936), but the fate of the others shows the provisional nature of any generalization about the proteolytic enzymes.

Grassmann distinguishes two main groups of proteolytic enzymes, the proteinases and the peptidases, which attack respectively proteins and peptides. The types were first distinguished in plant biochemistry in the pioneer work of Vines (1903–10), who named them peptase and ereptase. The proteinases are divided into three main groups, the pepsinases, whose optimum reaction is about *p*H 2, the papainases, with optima between *p*H 4 and 7, and the tryptases, with optima in the region of *p*H 8·9. The plant proteinases are mostly papainases, though insectivorous plants may contain enzymes of the pepsin and trypsin types. The peptidases may also be subdivided, as there are amino-polypeptidases, which attack only peptides in which there is a free amino group, and carboxy-polypeptidases, which require the presence of a free carboxyl group, and there may be special enzymes

splitting peptides which contain leucine, tyrosine or proline. The ability of a peptidase to split a given peptide is affected by two main factors, the number of peptide linkages in the molecule and the nature of the amino-acid residues.

Enzymes of the papain type are activated by hydrocyanic acid and by compounds containing the –SH group. A number of recent workers (Hellerman & Perkins, 1934; Purr, 1935*a*, *b*; Bersin, 1935) have concluded that the enzymes of the papain type contain a readily oxidized –SH group and that they are active only in the reduced form. A proteolytic enzyme from pea seeds has been shown (Blagoveshchenski & Korman, 1934) to be inhibited by cysteine, though Rondoni & Pozzi (1933) found that a similar enzyme of animal origin was activated. The activators mentioned above presumably reduce the oxidized form of the enzyme. Bersin writes the relation between the two forms of papain

$$2\text{PaSH} \underset{+2\text{H}}{\overset{-2\text{H}}{\rightleftharpoons}} \text{PaS.SPa},$$

on the lines of the transformation of glutathione from the reduced to the oxidized form. Urease is believed to be of a similar chemical nature. The relations between thiol compounds and enzymes have been summarized by Bersin (1935).

THE DEAMINATION OF AMINO-ACIDS

The *o*-quinones produced by the oxygenases of potato and *Lactarius vellereus* acting on aromatic compounds with an *o*-dihydroxy grouping can deaminate amino-acids, apparently according to the equation:

$$\text{catechol (or other compound with the } o\text{-dihydroxy grouping)} + O_2 \xrightarrow{\text{oxygenase}} o\text{-quinone} + H_2O_2$$

$$o\text{-quinone} + R.\text{CHNH}_2 \longrightarrow CO_2 + NH_3 + R.\text{CHO}.$$

The mechanism may be important in those plants in which oxidizing systems of this type occur (M. E. Robinson & McCance, 1925; Happold & Raper, 1925).

An enzyme deaminating amino-acids and peptides *in vitro* has been found in the juice pressed from a number of flowers by Grassmann & Bayerle (1934), but the mechanism of its action is not known.

The well-known suggestion that α-amino-acids are oxidatively

deaminated with the formation of α-keto-acids has been confirmed by Krebs (1933*a*, *b*) using kidney tissue. From alanine pyruvic acid was formed, from phenylalanine phenylbutyric acid and from α-aminobutyric acid α-ketobutyric acid:

$$R.CHNH_2.COOH + O = R.CO.COOH + NH_3.$$

Kidney tissue normally breaks down aspartic and glutamic acids without the formation of measurable amounts of keto-acids, but if the process is slowed by the addition of arsenious acid α-ketoglutaric acid can be isolated from the products of glutamic acid breakdown, and oxalacetic acid from those of aspartic acid:

$$\underset{\text{glutamic acid}}{HOOC.CH_2.CH_2.CHNH_2.COOH} \rightarrow \underset{\alpha\text{-ketoglutaric acid}}{HOOC.CH_2.CH_2.CO.COOH}$$

$$\underset{\text{aspartic acid}}{HOOC.CH_2.CHNH_2.COOH} \rightarrow \underset{\text{oxalacetic acid}}{HOOC.CH_2.CO.COOH}$$

The keto-acids thus formed could be decarboxylated by carboxylase, whose existence has been demonstrated in a number of higher plants by Zaleski & Marx (1913), and confirmed in barley by work done in this department (I. P. Norval, unpublished). The final products of deamination would then be carbon dioxide and aldehydes and acids with one carbon atom less than the original amino-acid:

$$R.CHNH_2.COOH \rightarrow R.CO.COOH \rightarrow \underset{\substack{+ \\ CO_2}}{R.CHO} \rightarrow R.COOH$$

Klein & Steiner (1928) and Steiner & Löffler (1931) found that nearly all of the ninety-nine species they examined gave off gaseous ammonia from the leaves and especially from the flowers. Many of them also gave off volatile amines, including methylamine, trimethylamine, amylamine and isobutylamine, which appear to play an important part in the scents of many flowers. The amount of ammonia lost in this way is not great—the authors suggest 3×10^{-6} g. per 24 hours per g. fresh weight—but it may be enough to account for the ammonia content of the atmosphere.

The production of amines in flowers is particularly interesting, as they are ephemeral organs in which rapid protein breakdown may be expected to take place. Klein & Steiner (1928) suggest that the long-chain amines such as amylamine and isobutylamine are formed by the decarboxylation of amino-acids:

$$R.CHNH_2.COOH \rightarrow R.CH_2.NH_2 + CO_2.$$

Trimethylamine might be formed from choline, which is a constituent of lecithin and is also found free in plants (Klein & Linser, 1933):

$$\underset{\text{choline}}{(CH_3)_3N.OH.CH_2.CH_2OH} = \underset{\text{trimethylamine}}{(CH_3)_3N} + \underset{\text{glycol}}{CH_2OH.CH_2OH}$$

The fate of the various barley proteins on malting has been studied by Bishop (1929*b*), who found a considerable decrease, during the second 100 hours after the beginning of steeping, in the content of the salt-insoluble proteins (hordenin and glutelin), and a corresponding increase in the salt-soluble compounds which include globulins, albumins, proteoses, peptides and amino-acids. Schjerning (1914) states (bringing his terminology into line with that of Bishop) that the sequence

insoluble protein → globulin → albumin → peptides → amino-acids

occurs in germinating barley. His results, together with those of a number of earlier workers, have been summarized by Lehmann & Aichele (1931). So far as our present fragmentary knowledge goes, the major stages of protein degradation may be summarized as follows:

Insoluble proteins
↓
Globulins
↓
Albumins
↓
Peptones
↓
Amino-acids
↙ ↓ ↘

Aldehydes + ammonia (deamination by oxidase systems)	Amines + carbon dioxide	Keto-acids + ammonia

THE REGULATION OF PROTEIN FORMATION AND BREAKDOWN IN THE PLANT

In some plant tissues, such as ripening seeds or young leaves, protein synthesis predominates, in others such as old leaves and germinating seeds marked hydrolysis occurs, and in many, such as mature leaves, these opposite processes go on together at more or less balanced rates. The factors controlling the synthesis and breakdown of protein have been investigated by a number of workers, but there is as yet no general agreement as to their nature.

The age of a tissue is known to have a marked effect on its protein metabolism, young leaves accumulating protein, while in older ones there is rapid hydrolysis. Deleano & Andreesco (1932) have shown that in *Salix fragilis* mature leaves lose half their total nitrogen, which returns to the perennating parts of the plant, between the end of their active period and leaf-fall. Combes & Echevin (1927) and Echevin (1931) find that in 3 weeks before leaf-fall beech leaves lose 50–80 per cent of their nitrogen. In herbaceous plants leaves formed during the early part of the growing season become senescent before its end and lose much of their nitrogen, which is translocated to the growing points, where new tissues are still being formed. Thus in different parts of the plant there are tissues of all metabolic ages, the materials used in building new tissues coming in part from the ageing ones.

Mothes (1933*a*) infiltrated young nitrogen-starved leaves of *Phaseolus* and *Nicotiana* with amino-acid mixtures corresponding to those formed by the hydrolysis of their proteins and found that rapid protein synthesis took place. He found that with such leaves a high concentration of oxygen in the surrounding atmosphere favoured condensation while a low concentration favoured proteolysis, and suggested that the stimulating effect of light on protein formation is due to the oxygen formed in photosynthesis, though it might equally well be due to the formation of active carbohydrates capable of taking part in amino-acid formation. Mothes's work has been extended by T. Schulze (1932) who has found in plants a substance, apparently chemically similar to cysteine, which is a redox body (i.e. capable of alternate oxidation and reduction) and activates proteolysis in the reduced form and inhibits it in the oxidized form. This work is particularly interesting in view of the recent results, mentioned on p. 46, on the activation of enzymes of the papain type by compounds with a free —SH group. Schulze calls his substance, which is not species specific, a mutator and ascribes to it the role of a regulator of protein metabolism. An activator obtained by Grassmann (1935) from papain preparations is of the same chemical type, being a peptide of cysteine and glutamic acid.

The work of Mothes and of Schulze has been heavily criticized by Paech (1935) who states that the rapid breakdown of protein in the absence of oxygen is due rather to damage to the cells than to any specific activation of enzymes. He admits that extracted enzymes are affected by oxidation and reduction, but is unwilling to extend this to the processes going on in the cell. Paech supports the older

view that the concentrations of active hexose and ammonia in the cell regulate the rates of protein formation and breakdown. It is known that in general protein breakdown is retarded by large supplies of carbohydrate, though Mothes (1933*b*) states that in old tobacco leaves rapid protein breakdown may occur in spite of a high starch content. In seedlings, too, there is rapid proteolysis in the presence of large carbohydrate reserves, but protein formation is also going on, though from amino-acids produced from stored protein. This synthesis will not be affected by the supply of carbohydrate.

Experiments by a number of workers (Vickery *et al.* 1933; Yemm, 1935) have shown that carbohydrates are preferentially respired, but that in carbohydrate shortage most of the carbon dioxide of respiration comes from substrates other than carbohydrate, which later work by Yemm (1937) has shown most probably to be protein. A study of the respiratory quotients of detached leaves shows that only for a very short time is carbohydrate alone respired, but the utilization of protein does not become important till most of the available carbohydrate has disappeared. The carbon dioxide produced in the respiration of protein is probably due to the decarboxylation of α-keto-acids formed by the deamination of α-amino-acids.

The further breakdown of the products of protein hydrolysis and of glycolysis may proceed on similar lines, and it may be that the inhibition of protein breakdown in the presence of carbohydrates may be due to competition for enzymes. Yemm (1936) has suggested that competition for active oxygen produced by oxidases may determine the relative rates of the two systems, while James has pointed out that both types of catabolism involve carboxylase, which is known (Zaleski, 1914) to act more rapidly on pyruvic than on any other α-keto-acid. In the presence of carbohydrate the formation of pyruvic acid may prevent carboxylase from acting on the other α-keto-acids formed by the deamination of α-amino-acids.

The work of Michael (1935), who was primarily concerned with the relation between the rates of protein and chlorophyll breakdown in senescent leaves, provides incidentally further data on the effect of different supplies of oxygen on protein hydrolysis in the detached leaf. He found, working with *Tropaeolum*, that in pure oxygen both protein and chlorophyll breakdown, which in general he found to follow parallel courses, were accelerated, a result which is difficult to reconcile with Mothes's theory, but which is in agreement with Yemm's suggestion. Michael concludes that yellowing is specially

connected with the removal of protein breakdown products, whether by oxidative deamination in the detached leaf or by translocation in the leaf on the plant. Conditions which slow yellowing, such as infiltration with glucose or an atmosphere with 7 per cent oxygen, also retard protein breakdown.

The whole question of regulation is bound up with the difficult one of the effects of the age of tissues on their metabolism. It is possible that in the leaves in which protein breakdown occurs in spite of abundant carbohydrate there is a breakdown of enzymes, so that the carbohydrate cannot be used. Or the change from synthetic to catabolic processes may be due to a change with age in the colloidal state of the protoplasm. The problem is not easy to study on purely chemical lines, as the reactions involved are probably not directly reversible so that the Law of Mass Action cannot be applied, and we can only measure gross concentrations of metabolites, while the course of the reactions is presumably determined by their concentrations at enzyme surfaces.

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PSEUDOMALLOMONAS ANGLICA: A NEW BRITISH FLAGELLATE

BY NELLIE CARTER, D.Sc.
The Botanical Department, Queen Mary College, University of London

(With 22 figures in the text)

IN the early months of 1934 a small motile chrysomonad was present in great quantities in a certain pond in Richmond Park. The organism has been observed subsequently in the two winters following. The individuals multiplied rapidly by vegetative division and very rich samples were obtained for study. The chief associates were *Chrysococcus rufescens* Klebs and *Scourfieldia complanata* West. In February 1935, the organism began to encyst and millions of cysts were present in the mud. Motile individuals were not present in such large quantities after this time. The organism is believed to be a new one and is described under the name of *Pseudomallomonas anglica* sp.n.

P. anglica is about $10 \times 6\,\mu$ and has a delicate transparent and colourless envelope which is elliptical in shape, and has a short but distinct neck at the anterior end (Fig. 1). With ordinary high powers of the microscope this envelope appears to be rough on the outside and netlike areolations may be distinguished with difficulty in surface view. The use of the oil immersion lens reveals the rhomboidal or slightly hexagonal areolations more clearly, and the uneven outline of the envelope is seen to be due to this structure of the membrane (Fig. 22). The collar of the envelope is about $2\,\mu$ in diameter and seems to show four rather indistinct apical teeth, but there is always a little mucilage there which renders observation rather difficult. The envelope is not affected by methylene blue, but Delafield's haematoxylin may stain it faintly in a few individuals and bring out the four-pronged collar more clearly (Fig. 22).

The envelope appears to be quite brittle and pieces of it frequently get broken away. It disappears completely on incineration and therefore contains no silica. The organism was being devoured in great numbers by a certain rotifer, but the envelope did not withstand digestion, and disappeared completely during its passage

through the digestive tract. This also tends to show that there is no strong silicification of the membrane. The envelope in the genera *Mallomonas* and *Pseudomallomonas* is believed to be covered with small silicified plates. The application of hydrochloric acid to the envelope of *P. anglica* does not cause the apparent plates to separate, however; the whole envelope slowly becomes more hyaline, and the markings gradually lose their definition as it slowly dissolves. The envelope does not dissolve with this treatment any more rapidly than the protoplast. It is presumably of an organic nature.

Conrad (1927, p. 499) has described the membrane of *Pseudomallomonas* as being similar to that of *Mallomonas*, and it is implied that silicified plates are a feature of both genera. In a later work (Conrad, 1933) he abolishes the genus *Pseudomallomonas* and includes all its species in *Mallomonas*. Conrad may be correct in believing that there is no justification for retaining *Pseudomallomonas* on the basis of the features by which it was first distinguished; viz. possession of a collar and absence of spines on the membrane. The question arises, however, whether there is any fundamental difference between the two genera in the nature of the envelope. The organism here described appears to have no silica in its membrane, and although it exhibits delicate rhomboidal markings, these do not seem to represent separate plates. It would be of interest to know whether this relatively simple kind of envelope is common to the other species of *Pseudomallomonas* previously described, in contrast to the more elaborate membrane of *Mallomonas*.

The organism has the power of renewing its envelope if necessary, and fragments of an older membrane may often be seen adhering to the exterior of a new and intact envelope.

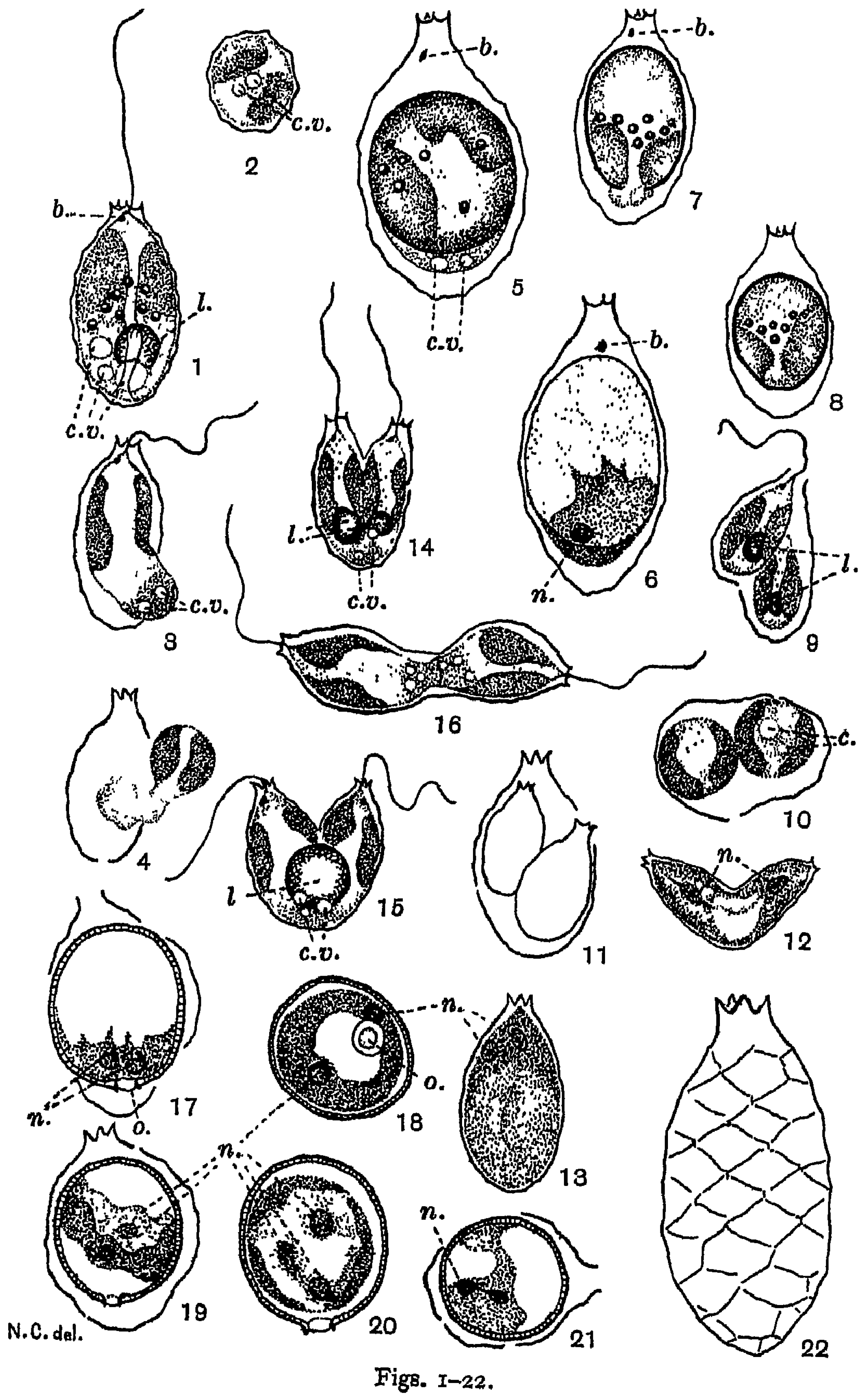
The protoplast nearly fills the envelope except at the anterior end where it is slightly attenuated and ends in the flagellum (Fig. 1). It has two massive pale yellow plastids in a parietal position. There is no pyrenoid and no stigma. The flagellum is 1–1½ times the body length and there is a prominent basal granule which appears pale blue by refraction (Fig. 1, *b.*). The nucleus is in the upper half of the organism and the basal part of the protoplast is characterized by two or three contractile vacuoles seen in varying stages of enlarging and contracting (Fig. 1, *c.v.*). Products of metabolism are abundant in the form of a large rounded mass of leucosin just above the contractile vacuoles and a number of smaller rounded globules distributed chiefly in the lower half of the organism. The organism was observed sometimes to lose its flagellum and become amoeboid, escaping from

its envelope either by the opening at the apex, or by bursting through the sides of the envelope (Figs. 3, 4).

Multiplication was common by the simple fission of the organism. The relation of the daughter individuals to the old envelope is not always the same in this process. Occasionally the old envelope comes to contain two individuals, each of which secretes an entirely new envelope for itself within the old one (Figs. 9–11). More often, however, each of the two daughter protoplasts separating by fission gradually develops the missing part of its envelope (Figs. 12, 14–16). Division in this case begins at the anterior end and for a time the posterior organs, contractile vacuoles, leucosin mass, etc. are only single and are shared between the two (Fig. 15). Later, as the new individuals become more perfect, the posterior structures are duplicated (Figs. 14, 16). The two daughters lie at first at an acute angle, but in the later stages are almost in a straight line, attached by their posterior extremities (cf. Figs. 15, 16). Division occurs in the active condition, the daughters developing their flagella very early. Where two individuals are formed in the same envelope, locomotion is effected by the movement of the flagellum of one of the organisms which protrudes from the mouth of the envelope, the flagellum of the second individual not being free (Fig. 9).

Cysts were abundant and were examined both in the living condition and after staining with Heidenhain's haematoxylin (Figs. 5–8, 17–21). Cyst formation is preceded by the loss of the flagellum, and the slight expansion and rounding off of the contents (Figs. 5, 6). The basal granule seems to become detached from the protoplast and can be recognized for some time lying free near the collar of the envelope (Figs. 5–7, *b*.), though it has usually disappeared by the time the cyst is mature. The central part of the protoplast in the developing cyst is occupied by a large mass of refractive oil (Fig. 5). The cyst is spherical or nearly so, and its wall is laid down with its opening at the posterior end of the organism (Figs. 17, 19). This inverted position of the cyst seems to be unusual. The species of *Pseudomallomonas* in which the cysts are known have the neck and plug of the cyst at the anterior end of the organism (cf. *P. Allorgei* (Deflandre, 1932) and *P. Playfairi* (Conrad, 1927, t. II, f. 59, 60)). The species of *Mallomonas* also have the cyst correspondingly orientated.

The mature cyst has a smooth and colourless wall, and reveals no obvious opening in the living condition; only a slight flattening of its rounded form at the posterior extremity shows where the



Figs. 1–22.

opening is situated (Fig. 8). In the young developing cyst the two plastids lie at the opening still in a parietal position, and a small quantity of residual protoplasm, which appears rather denser, can be seen entering the cyst from the outside (Figs. 5, 6). The contents at the anterior end are colourless, refractive and homogeneous, owing to the presence of oil. In the oily contents a large number of small colourless globules remain, usually lying in a ring at the upper margin of the chromatophores (Fig. 7). These globules and the oil are dissolved out in the processes of staining and mounting, and are therefore not shown in Figs. 6, 17–21. During cyst formation the envelope remains intact, and owing to the slight increase in the bulk of the contents it usually becomes rather pear-shaped in contrast to the elliptical vegetative individual (Figs. 5–8). If mud containing the cysts is allowed to dry, the envelope collapses on to the firm wall of the cyst, and may eventually disappear.

Although in the living cyst it is rare to find any trace of an opening or plug, in stained specimens mounted in canada balsam both can easily be distinguished. The wall of the cyst in these dehydrated individuals shows a very fine pitting and the plug appears highly refractive in contrast to the rest of the wall. The neck of the cyst seems to be merely a thickened rim in the wall (Fig. 18).

Staining shows that the nucleus is posterior in close proximity to the plastids in the young cyst (Fig. 6). As the cyst becomes mature, the plastids and the associated nuclei ascend higher up the cyst, and are usually seen to lie in the region of the equator, forming a parietal ring of stainable material (Figs. 18, 19). The rest of the interior,

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Fig. 1. Living individual. Fig. 2. Posterior view of smaller specimen. Figs. 3, 4. Organism actively amoeboid. Fig. 5. Early cyst formation, living. Fig. 6. Similar stage, fixed and stained with Heidenhain's haematoxylin. Fig 7. Older cyst, living, a little residual protoplasm still entering. Fig. 8. The mature cyst, living. Fig. 9. Dividing specimen. Fig. 10. Similar specimen, posterior view. Fig. 11. Old envelope with contained daughters. Fig. 12. Dividing individuals, stained. Fig. 13. Early division stage, nucleus divided, stained. Figs 14–16. Dividing specimens, living. Fig. 17. Young cyst, stained. Two nuclei, plastids, etc., posterior. Fig. 18. Posterior view of cyst, showing the opening and plug and the annular contents with two nuclei. Fig. 19. Mature cyst with two nuclei and plastids in an equatorial ring. Fig. 20. Cyst showing two nuclei and a third in process of division. Protoplast somewhat contracted and displaced during the processes of fixing and staining. Fig. 21. Division of the nucleus within the cyst. Fig. 22. Structure of the envelope. Figs. 6, 12, 13, 17–21, fixed and stained Heidenhain's haematoxylin. Figs. 1, 2, 5, 6, 12, 13, 17–21, ×1640. Figs. 3, 4, 7, 9–11, 14–16, ×1350. Fig. 8, ×1100. Fig. 22, ×3125. *b.* basal granule; *c.v.* contractile vacuole; *l.* leucosin; *n.* nucleus; *o.* opening of cyst.

except presumably a very thin lining layer of protoplasm, is occupied by oil in the living cyst. The plastids and nuclei sometimes ascend above the middle region of the cyst.

It is indicated that some nuclear changes take place in the cyst, since by the time the plastids have taken up an equatorial position, or even earlier, there are at least two nuclei (Figs. 17–19), and probably there is further division of the nuclei, since more than two nuclei are sometimes present (Fig. 20). Unfortunately the germination of the cysts has not been observed.

The organism is usually about 10–14 × 6–7 μ, but individuals may be as small as 8 × 5 μ, or as large as 20 × 8 μ. The cysts are usually spherical, or very slightly longer than their breadth, and are about 10 μ in diameter. The areolations of the envelope are about 3 × 1·5 μ. *Pseudomallomonas anglica* is near to *P. Playfairi* Conrad 1927 (= *Mallomonas Playfairi* Conrad, 1933, p. 17) in size, but differs in being less rounded at the extremities, in having a four-fid collar and in possessing two chromatophores. It is similar to *Pseudomallomonas Allorgei* (Deflandre, 1932) in form, but its dimensions are altogether smaller, and the scales of the envelope are smaller and lack the ornamentation described by Deflandre. Moreover the cyst has different characters. From *Pseudomallomonas elliptica* Kisselew (= *Mallomonas elliptica* (Kiss.) Conrad, 1933, p. 17) it differs in the much smaller dimensions of its envelope and plates and in its longer flagellum. It resembles *Pseudomallomonas bernardinensis* Chodat (= *Mallomonas bernardinensis* (Chodat) Conrad, 1933, p. 70) in general form but is somewhat broader at the ends and has not the imbricated scales. It also has a longer flagellum. Although similar in form to *Pseudomallomonas pyriformis* Valk. (= *Mallomonas Valkanoviana* (Valk.) Conrad, 1933, p. 34), and possessing, like this species, a toothed collar, it differs in its envelope, which has rhomboidal markings instead of elliptical scales. Furthermore it lacks a stigma (cf. Conrad, 1931, t. II, f. 47). The organism shows some resemblance to *M. oblongispora* Lemm. (see Conrad, 1933, p. 70) in size and in the annular nature of the contents of the cyst. Lemmermann's organism is, however, very imperfectly known, and the flagellate under consideration is therefore described as a new species. *Pseudomallomonas anglica* is probably of quite general occurrence. Besides the localities in Richmond Park it has been reported by Mr Scourfield from Epping Forest and is believed to occur in other parts of the country.

Pseudomallomonas anglica n.sp.

P. parvula, tunica elliptica quam lata sesquilongiore achroa tenuissima, areolis parvis *ca.* $3 \times 1\cdot5\,\mu$ signata quae in series spirales axi longiore transverse posito digeruntur, anteriore parte in collum breviusculum obscure quadrifidum evadente, corpore tunicam ferme replente, duabus chromatophoris praedito, flagello 1–1·5-plo longitudinem corporis superante, nucleo anteriore et vacuolis contractilibus pluribus posterioribus instructo et cystide sphaerica raro subelliptica in partem tunicae posteriorem aperta.

Habitat in stagnis, Richmond Park, Surrey.

Length 8–20 μ; breadth 5–8 μ; diameter cyst 10 μ.

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STUDIES IN THE AUTECOLOGY OF *CLADIUM MARISCUS* R.BR.

PART III. THE AERATION OF THE SUBTERRANEAN PARTS OF THE PLANT

By VERONA M. CONWAY

(With 4 figures in the text)

CONTENTS

Introduction

It has generally been assumed that the abundant air spaces in the tissues of aquatic plants make it possible for the submerged parts to obtain the oxygen necessary for respiration even though the surrounding medium does not yield a sufficient quantity. The air-space system is assumed to be continuous throughout the plant and of such a nature as to allow gases to diffuse according to the concentration gradients at a rate sufficient to supply the oxygen demands of all the tissues. These assumptions do not seem to have been put to a direct experimental test, although several pieces of work bear on the problem from one side or another.

There are researches such as those of Bergmann on the effects of oxygen lack on the roots of different types of plant and the relation of these effects to ecological distribution. Bergmann (1920) worked with eight or nine species, some of them land plants, for instance *Pelargonium*, *Impatiens balsamina* and *Zea*; some of them hydrophytes, for instance *Ranunculus abortivus*, *R. sceleratus* and *Cyperus alternifolius*. He found that with the land plants, submergence of the soil in which they were growing caused a decrease in water absorption by the roots and finally killed them. These effects could be prevented by aerating the water. The hydrophytes, however,

showed better growth of the entire plants when grown in submerged soil. This difference in behaviour Bergmann attributed to the presence of aerenchyma in the hydrophytes but he does not discuss the aerenchyma further. He also quotes the work of several other people on the effects of poorly aerated soil, and the results differ according to the species, though agreeing in the main with his conclusions.

The most extensive work along these lines has been carried out by Cannon (1925) and the range of variation from species to species can be judged from his results. His researches covered some thirty species of very varied type. He measured the actual growth in length of roots under various oxygen concentrations and compared it with the growth under "normal" conditions, i.e. moist soil with a soil atmosphere of approximately atmospheric composition. He found that the effect of a given oxygen deficiency varies according to the temperature and he gives his results as critical oxygen concentrations for any particular temperature. The lower critical concentration is that at which growth ceases, the upper is the lowest concentration at which growth is as rapid as under "normal" conditions. With all species it was found that the critical concentrations were lower the lower the temperature. The observations are somewhat scattered and only in the case of one or two species do they cover a range of temperature. Often, too, the oxygen concentrations are too widely separated, and the individual variation of the plants too great, to allow a definite determination of the critical concentrations. Table I shows a few of his results, with *Oryza sativa* and *Salix lasiolepis* as the most tolerant to oxygen deficiency and *Phaseolus* as the least tolerant.

Table I

Species	Temperature ° C.	Critical concentrations %	
		Lower	Upper
Salix lasiolepis	21	—	Less than 1·6
	30	—	More than 2·0
Zea Mais	18	Less than 3·0	Less than 10
	22	Less than 3·0	—
	27	Less than 3·0	—
	30	Less than 3·0	More than 10
Oryza sativa	23	Less than 0·5	—
Phaseolus	Not given	More than 7, but variable	More than 10

Cannon (1932 *a* and *b*) has also carried out some experiments which deal with the question of oxygen supply to roots. They were designed to

find out whether oxygen liberated in photosynthesis could be a source of supply to the root system. He placed the root systems in water and measured the rate of loss of oxygen from the water when the shoot received various treatments. The plants used were *Salix laevigata* and *Helianthus annuus*, grown in culture solution. He found that in about two-thirds of the experiments less oxygen was taken by the roots from the external medium when the shoot was in bright light than when it was in the dark, and from this he concluded that in the first case the root tissues were obtaining their oxygen from a source inside the plant. The remaining third of his results he tried to explain on the ground that the temperature in the bright light was sometimes higher than in the dark and so caused an increase in root respiration which masked the fact that extra oxygen was being supplied from the shoot. The explanation does not by any means hold for all the results which do not agree with his main conclusions, so that the latter must be regarded with considerable reserve. The question of how the oxygen is to pass from the leaves to the root is treated very scantily, the suggestion being thrown out that it moves downwards in solution, against the direction of the transpiration stream.

Actual analysis of the gases in the intercellular spaces of plants has been carried out in several cases. Barthelmy (1874) carried out a number of experiments along these lines, using hydrophytes, with a view to showing that their air-space system allowed the diffusion of gases from one part to another. He extracted the gases by reduced pressure and analysed them, and showed also that gases could be forced by pressure to move along the air channels. He worked almost entirely, however, with leaves and petioles and so did not supply much information as to the aeration of roots and rhizomes. Perhaps the most interesting result he obtained was with *Nelumbium speciosum*, where he caused bubbles of gas to be emitted by one leaf when he submitted another to increased pressure, indicating the continuity of the air-space system through the stem to the leaves. His analysis of the gases from *Nelumbium* leaves showed a variation in oxygen from 17 to 24 per cent, the latter value being obtained in the evening; after prolonged immersion (whether in light or dark is not stated) he found an oxygen concentration as low as 10 per cent. In these, as in other experiments, he found nitrogen percentages higher than 79 per cent, which he attributed to the more rapid diffusion of nitrogen through small apertures. Barthelmy also suggests that the fleshy aerenchymatous roots of plants such as the

Cyperaceae take gas from the surrounding mud or water. This gas is at more than atmospheric pressure and passes up into the leaves and out through stomata. He gives no evidence, however, that such an excess pressure exists, nor that the gas is able to pass from the roots to the rest of the plant.

A summary of early analyses of gases from intercellular spaces of plants is given by Barthelmy and reproduced in Table II. All the results refer to plants under natural conditions and the high nitrogen percentages are referred to the higher diffusion coefficient of nitrogen compared to oxygen or carbon dioxide. It seems equally probable that it is due to the removal of oxygen by respiration and the solution of some of the carbon dioxide produced.

Table II

		Percentages		
Authority	Part of plant, etc.	N_2	O_2	CO_2
De Saussure	Branch of apple tree	86	9	5
Boussingault	Cherry laurel	88	6·65	5·34
Martins & Moitessier	Roots of *Jussiaea*	84 to 92	15 to 8	—
	,, *Aldrovandia*	84·5	15·5	—
	,, *Pontederia crassipes*	85·9	14·1	—
Dutrochet	*Nuphar lutea*: Rhizome	84	16	—
	Root	82	8	—
	Leaves	82	18	—

Dutrochet's results are especially interesting since they deal with different parts of the same plant. Apart from this work in 1837, there does not appear to have been any attempt to study the internal air-space system as a whole. The present article is an attempt to fill this gap for one species at any rate.

Two main types of experiment have been used in this attempt. The first aimed at showing directly whether or not the air spaces are linked into a continuous system, by measuring rates of flow of air under pressure from one part of the plant to another. This method also showed the relative ease of flow of gas along different paths. The second type was concerned with the composition of the gas in the internal atmosphere of the plant. By showing the interrelations of the compositions in different parts of the plant, under varying external conditions, an attempt was made to find out the paths along which oxygen could diffuse as a result of a concentration gradient.

From investigations of this kind it may be possible to judge how far the presence of abundant air spaces is a necessity for the existence of the plant in its normal habitat, waterlogged soil. They should show experimentally whether it is justifiable to speak of this particular

anatomical feature as an "adaptation" rather than as an effect of the habitat, or an incidental specific character.

EXPERIMENTS ON MASS FLOW OF GAS

The principle of the method used is the same as that used in measuring stomatal opening with a porometer. A small negative pressure is applied to the cut end of one part of the plant, usually a root or rhizome, by sealing it to a tube leading to a vertical glass tube down which a small thread of mercury is allowed to fall. The rate of fall of the mercury thread gives a measure of the ease with which gas can pass out of the plant at this point. The gas drawn out here is replaced by air which enters the plant at some other point, and the place of entry of the air can be controlled by immersing the rest of the plant surface in water.

The length of the mercury thread was about 7 mm. so that the pressure above it, when it was near the top of the vertical porometer tube, was only 99 per cent of atmospheric pressure. Before starting the experiments, the porometer tube was calibrated by using it to measure the rate of flow of air down a piece of capillary tubing about 1 m. long. Then if later the mercury thread were accidentally lost, the length of the new thread could be adjusted to give the same rate of flow through the same capillary tube. The ordinary flanged cup and plate of the porometer were not suitable for making connexion with the plant in these experiments. When air was to be drawn out of a root or rhizome a short glass tube was connected to one limb of the porometer and passed through a cork into a glass tube 2 cm. in diameter and 5 cm. long. The open end of the latter was closed by a lump of plasticine through the centre of which passed the free end of the root or rhizome. The plasticine could be pressed down to give an airtight joint with the glass tube; the surface of the root or rhizome was painted over with xylol and the plasticine could then be sealed round the surface. The junction was painted over with luting wax to ensure that it should be airtight. In the case of the rhizome, the scale leaves had to be removed over a length of 1 cm. or so, in order to make an airtight seal possible. Fig. 1 shows these arrangements.

The simplest experiments were those where air entered at one end of a piece of root cut from the plant and was drawn out at the other end. The wide glass tube with the plasticine joint was immersed in water so as to leave only the free end of the root projecting into the air. Isolated lengths of rhizome were similarly treated.

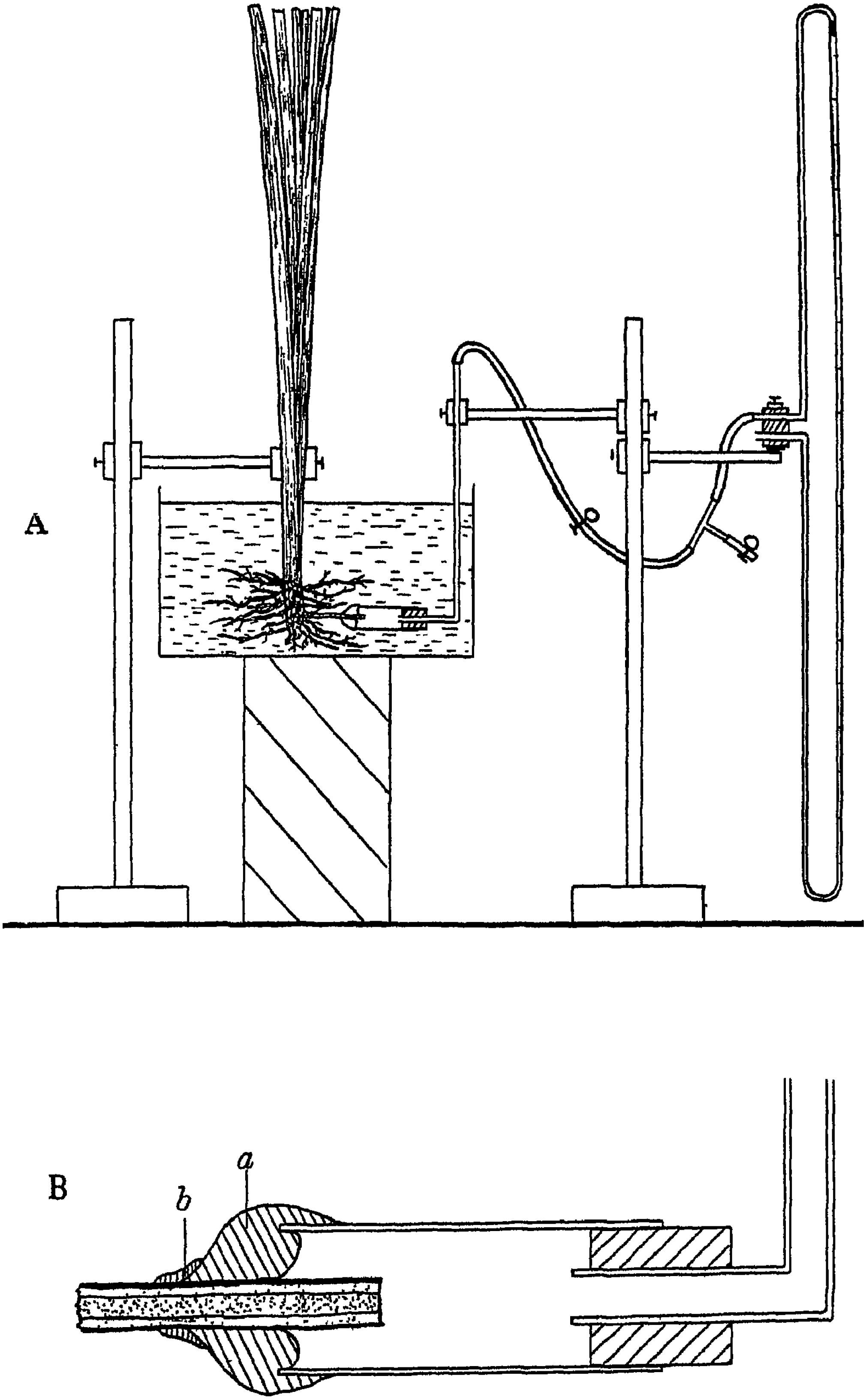

Fig. 1 A. General arrangement for porometer experiments when air is to be drawn out through the rhizome or root, and enter the plant through the leaves. B. Enlarged drawing to show in detail how the porometer is attached to the rhizome or root. *a* plasticine, *b* luting wax.

When air was to enter the plant through the leaves, the final arrangement of the experiment was that shown in Fig. 1 A. The wide glass tube was sealed on to the root or rhizome while the plant was clamped in the air, and the water bath was then brought up so as to immerse all the lower parts of the plant. For practical convenience the upper parts of the leaves were cut off, except in certain cases, leaving not more than 50 cm. of lamina above the base. This meant that the interior of the leaves was open to the air in all but the very youngest type B leaves[1] and hence the question of stomatal resistance need not be considered.

In a few cases the porometer was attached to a root and air entered the stock through the fresh surfaces left by cutting off the leaves below the growing point, and cutting off the oldest part of the stock below the insertion of the root. In all experiments using roots it was of the greatest importance to select those with an unbroken surface. Apparently intact roots are very often damaged close to the stock, and at first experiments were frequently vitiated owing to this cause.

A slightly modified method of attachment to the porometer was used to measure rates of flow through the internal spaces of the leaves alone.

Readings were made of the time taken for the mercury thread to pass through the upper five divisions of the porometer tube. These divisions are 2·5 cm. apart. If the rate was exceedingly slow the time for one division was measured. In all cases, after the first reading had been taken, the seal between the glass tube and the plant organ was reinforced or renewed, and if the next reading showed a slower rate it was taken to mean that the first seal had been faulty, and the alternate taking of readings and re-sealing were repeated until a constant reading was obtained. The first seal was almost always found to have been satisfactory.

The results are expressed as rates of flow in units of $100/t$, where t is the time in seconds for the mercury thread to pass through one division of the porometer tube.

[1] The anatomy of the plant has been described in the first paper of this series (Conway, 1936). The leaf types are briefly as follows, passing outwards from the growing-point:

Type A. Not more than 5 mm. high, rudimentary.
Type B. Varying in height, but all growing at the same rate.
Type C. Still green, but growing slowly or not at all.
Type D. Withered throughout their length.

Root alone

Pieces of root varying in length from 3 to 7 cm. were used and the ends cut across cleanly. The roots used were of the fleshy type, all about 3 mm. in diameter, and the few small branches they bore were cut off at the surface of the parent root, which was then painted over with wax except at the cut end to prevent water entering when the root was immersed. The rates per centimetre of root, in the units defined above, were

743, 135, 186, 183.

The first result was for an older root in which the cortical tissues had broken down much more completely than in the others. The results are thus very variable, but all lie above 100 units.

Rhizome alone

The rate of flow down isolated pieces of rhizome was also determined, and the lengths used varied from 3 to 7 cm. The rates per centimetre of rhizome were

165, 1430, 286, 1650.

Again the variation is extreme and could be explained by differences in age of the rhizome. The cortical tissues were much more compact in parts of rhizomes near to an apical bud which had not yet developed into a shoot.

Leaves, stock, rhizome and root

Most of the remaining results have been collected together in the form of Table III. The figures referring to any one plant have been enclosed in one block and each plant is named by a letter for ease of reference. The experimental procedure is summarized on the left. The experimental treatment of plant T may be described in detail to make clearer the results given in Table III. The porometer tube was attached to a young rhizome and the general arrangement was that shown in Fig. 1. Readings were taken first of all with no water in the bath, and the seal was retouched until a constant value was obtained. Water was then poured into the bath so as to immerse the lower part of the plant, and a reading taken, which gave a value of 3·6 units. The bath of water was then removed, and the type D leaves were cut through with a razor about 2 cm. above the growing-point. Readings were taken with the plant in air until it was shown that the seal was airtight and then the base of the plant was immersed in water and a reading taken, which gave a value of 3·0 units for the

Table III

Air passes from	Condition of plants	Rates of flow of air							
		A	D	E	G				
Leaves into parent rhizome	No leaves blocked	0·7	0·3	0·6	1·0				
	Leaves cut shorter		(0·4)						
	Type D blocked				0·2				
			B	R	S	T	U	V	W
Leaves into young rhizome	No leaves blocked		0·8	6·5	5·4	3·6	8·1		
	Leaves cut shorter		(0·8)						
	Type D blocked			4·5	3·6	3·0	5·6	3·8	
	Types B and C cut shorter			(4·9)	(3·9)	(3·0)			
	Types C and D blocked			0·8	0·5	0·8	0·25	0·3	0·2
	Type B cut very short						(0·3)	(0·2)	(0·2)
				O	P	Q			
Leaves into root	No leaves blocked	0·5	1·5	1·8	1·1	2·4			
	Leaves cut shorter	(0·9)							
Stock into root	Leaves and growing-point removed				1·6	3·1	3·3		

rate. The cut ends of the type D bases were below the surface of the water so that air could not enter the plant through them. Table III indicates this condition as "type D blocked". The next procedure was to cut off about 30 cm. of the types C and D leaves that remained, so that the length of leaf through which air had to be drawn was very much reduced. A reading was taken again, and the rate (3·0 units) was unaltered, indicating that only a negligible resistance to air-flow was offered by the parts of the leaves that had been cut off. The results of cutting leaves short in this way have been put in brackets throughout Table III because the area through which air can enter the stock is not altered, but only the length of the path up to the stock. After this the water-bath was again removed and the type C leaves cut off close to the growing-point, exactly as had been done with the type D leaves before. The reading obtained after the water-bath had been replaced was 0·8 unit. This great reduction in rate indicated that a very large resistance had been introduced into the system by blocking up the type C leaves as well as the type D.

Plants R, S, and U were treated in precisely the same way and all gave the same results. With plant U, a further step was taken, in that the column of type B leaves which remained was cut off just above the water surface and another reading taken, giving a rate of 0·3 unit. This is only slightly higher than the previous rate (0·25 unit) and indicates that a large part of the resistance to the entry of air into the stock through the type B leaves is due to the meristematic region at their base, for, had this region been one of relatively small resistance, the removal of the rest of the lamina above it should have given a marked increase in rate. This result was confirmed with plants V and W where readings were only taken at the later stages of the experiment.

Plant B was considerably younger, and the first reading gave a rate of 0·8 unit, which is considerably lower than those obtained with the more mature plants R to W, and similar low rates were given by plants A, D, E, and G, which were all very young plants and had not yet produced daughter rhizomes. The porometer was attached to the parent rhizome, but otherwise the arrangements were the same as before. This lowered rate with younger plants is readily explained when it is remembered that they have not yet had time to produce type D leaves, or rather, that the latter are represented by only scale leaves a few cm. in length. Moreover all the green leaves are growing and therefore not fully differentiated at the base, so that it is only natural to find not only a generally lowered rate,

but also a further large drop in rate with plant G when the fully differentiated scale leaves were cut short and blocked with water.

With plants A, B, O, P, and Q, readings were taken with the porometer attached to a root instead of to a rhizome, and the base of the plant immersed in water as before. The values are variable, but not strikingly low compared with those obtained when air was drawn out through the rhizome, though lower values might have been expected when one considers the much smaller cross-sectional area of the root compared with the rhizome. This shows that there cannot be any very large resistance at the transition from root to stock.

In the case of plants P and Q, the stock was cut cleanly across above the root to which the porometer was attached, and after resealing the joint, readings were taken which showed a rate only slightly higher than before. In the case of plant Q a reading was also taken with another root, and the result (3·3 units) was close to the first. What had been done by this treatment was to remove the region of attachment of the leaves to the stock so that the tissues of the stock were in direct contact with the air. The results indicate that for the type C and D leaves at any rate this region is not one of high resistance. Hence the larger part of the resistance measured in the earlier experiments must be attributed to the compact nature of the tissues of the stock itself.

So far there has been little discussion of rates of flow of air through the leaf lamina itself. The figures in brackets in Table III show however that very little of the total resistance involved in the various experiments is removed by cutting off 30 cm. or so of all the leaves provided that their internal air spaces are open to the air at the upper end. In addition there remain for consideration the direct measurements of the resistance to flow along the length of the leaf. For this type of experiment, instead of the wide glass tube and plasticine of the previous experiments, a narrower glass tube, 1 cm. in diameter, was used, and into the end of this fitted a cork cut into two pieces of such a shape that they held the leaf lamina firmly between them but did not compress it. Ten-centimetre lengths of leaf were used; the pieces of cork were put in place round the piece of leaf near one end of the 10 cm. length and then inserted into the glass tube. The junction was sealed over with luting wax, so that air could only enter the tube through the tissues of the leaf. The surface of the projecting piece of leaf was painted over with wax, except at the cut end, to avoid the possibility that air should enter through the stomata. Measurements were made for different types of leaves, with

lengths taken from various heights, but the results all led to the same conclusion so that only those from two leaves will be given as illustrations. The first was a type C leaf; the rates are expressed in the same units as before, for 10 cm. length of leaf lamina:

0–10 cm. from base	100
10–20 ,, ,,	125
20–30 ,, ,,	125
30–40 ,, ,,	100
40–50 ,, ,,	82
50–60 ,, ,,	62
60–70 ,, ,,	50
70–80 ,, ,,	25
80–90 ,, ,,	12

The part of the leaf above 90 cm. was withered and was therefore discarded. A type B leaf was taken from the same plant and measurements were begun at a point on it which corresponded morphologically, as far as could be judged by the shape and width of the lamina, with the point where they had left off for the other leaf. Successive 10 cm. lengths were taken above this point until the rate of movement of the porometer became too slow to measure. The results were:

20–30 cm. above base	4·5 units
30–40 ,, ,,	2·4 ,,
40–50 ,, ,,	1·3 ,,
50–60 ,, ,,	0·84 unit
60–70 ,, ,,	0·24 ,,
70–80 ,, ,,	No movement of mercury thread

There is therefore very little resistance to the flow of air through the lowest 70 cm. of the type C leaves and hence of the type D leaves also, since there is no difference in the structure of the two types. Above this height, however, the resistance increases very rapidly. Thus in the experiments dealing with the flow of air from the leaves to the stock, little resistance can have been offered by the 50 cm. or so of the types C and D leaves which was the most that was present. Some of the type B leaves would be composed entirely of the upper, resistant part of the lamina and this is no doubt partly responsible for the great resistance to the entry of air into the stock through the type B leaves; the other cause is the presence of the meristematic region at the base as demonstrated by the experiments with plants U, V, and W (Table III).

In surveying and summarizing these results, three main points stand out:

(*a*) In all experiments where air is only allowed to enter the lower parts of the plant through the leaves, the rate of entry is very greatly

reduced if the older leaves are blocked and the type B leaves form the only channel through which air can pass.

(*b*) There is a striking contrast between the order of magnitude of the rates of flow through isolated pieces of root or rhizome and the rates obtained when the stock forms part of the system through which air is flowing. Rates in the latter type of experiment never exceed 10 units; in the former, they would not be as low as 10 units unless a considerable length of the organ was used.

(*c*) The high resistances found when the stock is involved are due to the tissues of the stock itself, and not to especially resistant regions at the junction between leaf and stock or between root and stock.

These conclusions fit precisely with the descriptions in Part I (Conway, 1936) of the different tissues and the variations between them with respect to the relative abundance of air spaces in them. To confirm and illustrate this agreement of experiment with expectation some measurements were made of the percentage volume of air space in the different tissues. Pieces of the tissues or plant organs were separated and weighed, and then injected with paraffin under reduced pressure and reweighed. Their external volume was measured by dropping the injected pieces into a small measuring cylinder of paraffin. The results were as follows:

	% volume air space
Rhizome (a length of about 1 cm.)	38
Stele of rhizome alone	6
Root (a length of about 2 cm.)	60
Base of type D leaf (a length of about 2 cm.) ...	56
Base of type C leaf (a length of about 2 cm.) ...	26
Base of type B leaf (a length of about 1 cm.) ...	6
Cortex of stock alone (several pieces, making a total volume about 0·2 c.c.)	35
Stele of stock alone (one piece about 0·5 c.c. volume)	6

Since the stele of the rhizome makes up more than half the volume of the rhizome, the proportion of air space to tissue in the cortex must be in the region of 4 : 1, to judge from the first two results.

The stele of the root is narrow compared with the total diameter. The 60 per cent of air space in the root is made up of the air spaces in the root cortex, since the stele of the root, except for the vascular bundles, consists entirely of close-packed fibres.

The stock cortex shows a rather higher percentage than one might expect from the high resistance to air flow that is shown by the stock as a whole. The explanation may be sought partly in the nature of the air spaces. In the leaves, the air spaces are in the form of longitudinal channels, for if pith cells are present in the central leaf

spaces, they are arranged roughly in longitudinal rows. The cells of the stock cortex, however, are not regularly arranged, so that gas has, so to speak, to wind its way in and out between the cells. Then

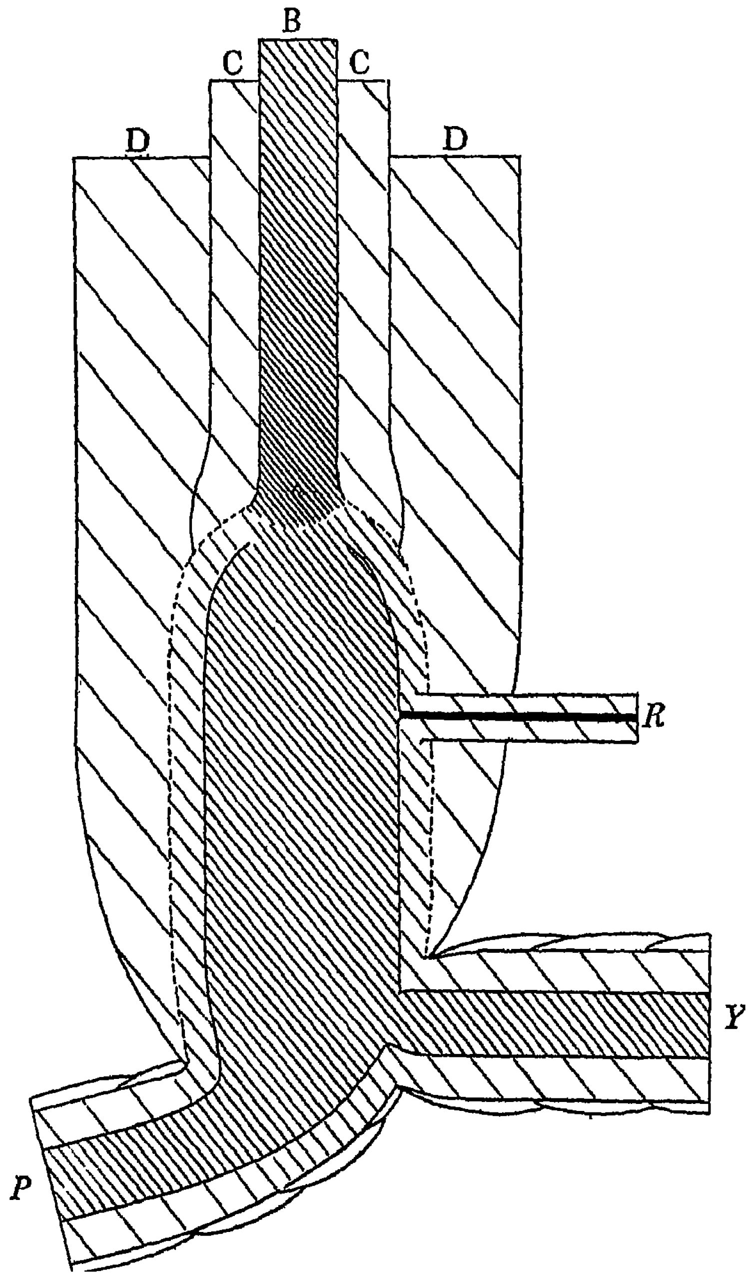

Fig. 2. Diagram to illustrate the results of the porometer experiments. The density of shading of a tissue is roughly proportional to the degree of resistance to mass flow of gas through it. B, C, D, leaf types; *R* root; *P* parent rhizome; *Y* young rhizome.

again, the cross-sectional area of cortex at any level of the stock is small compared with that of the whole stock, so that it is not surprising that the stock should appear to offer considerable resistance to the flow of air through it.

The other results accord with what would be expected from the preceding results; the small percentage volume in the meristem of the type B base, for instance, may be compared with the high resistance to the flow of air from type B leaves into the stock.

The general conclusions arrived at so far are expressed in the form of the diagram in Fig. 2.[1] The resistance to mass movement of gas through the various parts of the plant which are below the ground surface is indicated by the cross-hatching, which is denser the greater the resistance.

EXPERIMENTS ON DIFFUSION OF OXYGEN

The general aim of these experiments was to obtain some picture of the gas compositions in the intercellular spaces of the plant, but the particular method of approach was an attempt to discover how far the oxygen concentration in the internal atmosphere of the roots depends on the concentration in the medium surrounding the roots. Plants were therefore grown with their bases submerged in water which was in some cases devoid of dissolved oxygen; in other cases the oxygen concentration was in equilibrium with that of the atmosphere, or nearly so.

It was found that if a thick layer of peat was put at the bottom of a bucketful of water, the oxygen concentration in the lower layers of water fell to zero if there was no disturbance. This seemed a suitable method for subjecting *Cladium* roots to low oxygen concentrations, especially since it resembled to some extent the natural conditions of growth. The contrasting condition was obtained by having the roots in the surface layers of clean water.

Gas samples were obtained from various parts of the plants and analysed for carbon dioxide and oxygen with a Bonnier and Mangin apparatus. This has been described in detail by Thoday (1913) and it was used according to the methods indicated in that paper. It is designed for analysing volumes of gas ranging from 0·2 to 1 c.c., and is supposed to have an accuracy of 0·1 per cent. The actual degree of accuracy attained in this work was considerably less than this; the matter is discussed later.

The first series of analyses extended over most of May 1934. About fifteen plants had been dug up from the Mixed Sedge vegetation of Wicken Fen. They were brought back to the laboratory as carefully as possible and divided into two sets. Those of one set (set 1) were

[1] Cf. Conway (1936), "Studies, etc.", Part I, Figs. 1 and 2.

placed with their root systems just below the surface of the water in a shallow wooden trough. Those of the other (set 2) were placed in galvanised iron buckets full of water in the bottom of which was a layer of peat about 1 in. thick. The root systems rested on this layer. Both sets of plants were out of doors but in a sheltered basement window. The plants did not vary much in age, as judged by the lengths of their stocks; these were from 3 to 5 cm. in height. A few

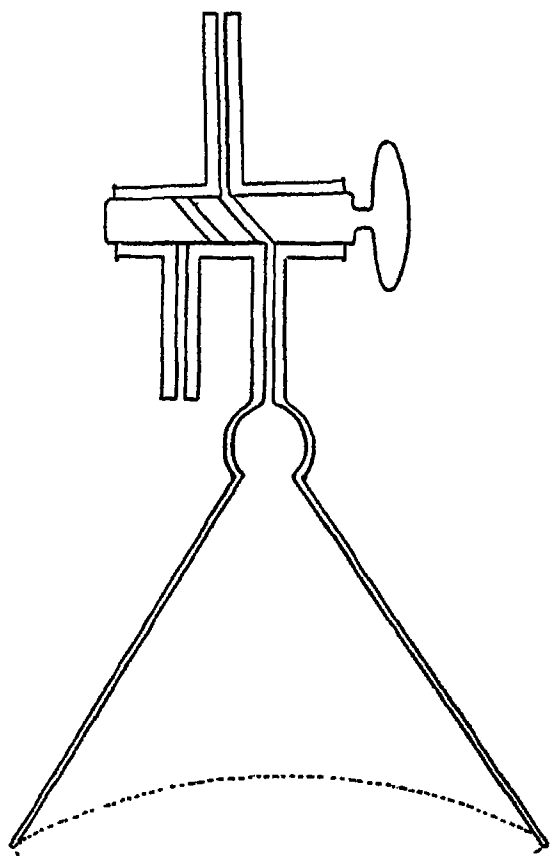

Fig. 3. Type of funnel used for obtaining gas samples in the analysis experiments.

plants died as a result of transplanting, but there were enough left to allow five plants from each set to be investigated.

The samples from any one plant were obtained early in the day and analysed during the rest of the day. Before anything was done with the plant, however, a sample of the water surrounding the roots was removed and the dissolved oxygen estimated by the Winkler method as described by Alsterberg (1926).

In obtaining the gas samples a glycerine-salt mixture was used which will be referred to as the G.S.M. It consists of a mixture of

equal parts of pure glycerine and saturated salt solution with a few drops of concentrated hydrochloric acid. It has the property that oxygen, carbon dioxide and nitrogen are, to all practical purposes, insoluble in it. The samples were introduced into special funnels, shown in Fig. 3; the funnels had been filled with G.S.M. and inverted in a bath of the same liquid. The gas which collected in the vertex of the funnel was then transferred by means of a mercury reservoir and three-way tap system to one of the sample tubes of the Bonnier and Mangin apparatus. When all the samples had been transferred in this way the analysis of each was carried out in turn.

(1) *Green leaves*

In most of the plants there was only one type C leaf and it was this one which was taken to yield a gas sample. Before moving the plant at all, the leaf was gripped firmly with the fingers about 20 cm. above its base and cut off below this point. A length of 20–30 cm. was then cut off above the gripped point, the cutting being done below the surface of the bath of G.S.M. It was then cut up into pieces 1–2 cm. long below one of the funnels, which was filled with G.S.M. and clamped, inverted, in a convenient position in the bath. The pieces rose to the top of the funnel, which was then transferred in a smaller dish of G.S.M. to an evacuating chamber. The pump had only to run for a few seconds before enough gas had been extracted from the pieces of leaf. The whole process was carried out as rapidly as possible because it was found on investigation that if the bits of leaf were left long, say an hour or more, in the G.S.M., they yielded a gas mixture much richer in carbon dioxide than at first, suggesting some abnormal effect in the leaf.

The only occasion when there was any departure from the procedure just described was on 1 May, when the leaf was taken after the rest of the plant had been dealt with, and not before.

(2) *Roots*

The whole plant was lifted out of the water in which it had been kept, and if the roots were muddy they were rinsed very rapidly under the tap, as much water as possible was shaken off, and the base of the plant immersed in the G.S.M. bath, which was deep enough to submerge the whole stock of the plant. The plant was held in place by a clamp. An intact root was gripped with forceps near its proximal end, and cut off close to the stock. The gas contained in it was squeezed out by pressure with the fingers below one of the

inverted funnels. To make this easier each portion of the root was cut off after it had been squeezed. Only intact roots were used because preliminary experiments had shown, as would be expected, that the oxygen concentrations in broken roots were higher than those in intact roots of the same plant when it had been in well aerated water. All the intact roots were dealt with in this way.

(3) *Leaf bases*

When the roots were finished with, the plant was unclamped and cut across with secateurs just above the growing point of the stock. The lowest 2 cm. was then cut from the basal ends of the leaves. All the cutting was done in the G.S.M. and neither the stock nor the pieces of the leaf bases were allowed to come into contact with air. The gas was obtained from these 2 cm. pieces by squeezing them under an inverted funnel. The bases of the type D leaves were brown whereas those of the younger leaves were colourless, so that they could be distinguished from each other and the gas from each type could be obtained separately if desired.

This method was not used in the first experiment (1 May), but instead, the needle of a hypodermic syringe was pushed into the leaf bases below the surface of the G.S.M. and a sample of gas drawn into it. The disadvantage of this lay in the uncertainty as to how far the needle had penetrated so that the gas sample was of rather doubtful origin, and the method was not used again in the other experiments.

In all cases care was taken to prevent debris from remaining in the funnels together with the gas samples since they might be evolving carbon dioxide, or otherwise influencing the gas composition.

(4) *Stock*

The stock, still kept immersed in the G.S.M., was now stripped as clean as possible of leaf bases and roots so as to make sure that no gas bubbles were obtained that did not come from the interior of the stock itself. Since the stock necessarily remained in the G.S.M. for some time, say up to half an hour, it was thought that it might show a reaction by an abnormal production of carbon dioxide. To minimize this, about 1 cm. was cut off from either end so that the internal tissues of the block which remained were less likely to have been affected by the abnormal conditions. The growing-point was removed by this procedure, so that the block was similar throughout its length. It was then squeezed below an inverted funnel. The stock is very hard and only yields a small volume of gas even under the maximum

pressure, which could be applied by the fingers. In the experiments of 11 and 15 May, where a horizontal rhizome was still attached to the stock, a short piece was taken with the stock to increase the volume of the gas sample.

The bath of G.S.M. at the end of these proceedings inevitably contained much plant debris so that it had to be filtered in a large funnel overnight. Also the plant roots were, of course, wet when introduced into the bath; this had to be compensated as far as possible. A quantity of salt was added which would saturate 1 c.c. of water, which was estimated as about the volume of water introduced. This was done every day before an experiment was started.[1]

The results of the first series of experiments are shown in Table IV. It shows the percentage carbon dioxide and oxygen concentrations for each sample. The results of the Winkler estimation of the dissolved oxygen in the water round the roots of the plants are expressed in milligrammes per litre. For water in equilibrium with the atmosphere, the value would be 10·1 mg. at 15° C., 7·6 mg. at 30° C. The air temperatures for the summer months when these experiments were performed probably lay for the most part between these limits.

The details of the results and also the limitations of the technique will be discussed when the second series of experiments has been described. It soon became clear, as the first series of experiments proceeded, that the root oxygen in set 2 was surprisingly high considering the low external concentration. The latter, however, was confined to the lower layers of the water, and there must have been more oxygen nearer the surface. Hence the water round the leaf bases was probably not so deficient in oxygen as that round the roots and possibly the relatively high oxygen values shown in the leaf bases of set 2 may have been the cause of the high root values. To test this, two plants had their type C and D leaves cut off carefully with a sharp knife about 3 cm. from their base, leaving the inner column of type B leaves intact. They were then immersed above the cut ends of the leaves in one of the buckets of peaty water. After a few days the root gas was analysed in the usual way and showed far lower oxygen values (2·29 and 3·06 per cent respectively) than any obtained in the preceding experiments. In view of these results, the second series of experiments was designed:

[1] To make certain that the G.S.M. was not absorbing appreciable quantities of oxygen, carbon dioxide, or nitrogen, a sample of expired air was analysed with a Haldane apparatus both before and after it had been bubbled through the G.S.M. several times. No alteration in composition was found.

Table IV. *Results of first series of analysis experiments. Carbon dioxide and oxygen are given as percentages of total volume, dissolved oxygen in water in mg. per litre*

Date	Dissolved oxygen in water	Leaves		Roots		Stock		Type D bases		Type C bases		Bases of all leaves		Types B and C bases	
		CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2
Set 1. Entire plants, in well aerated water:															
1 May	7·66	1·61	17·61	1·46	17·79	1·68	15·78	—	—	—	—	1·35	19·79	—	—
7 „	8·16	0·75	20·23	2·15	19·03	1·28	16·91	—	—	—	—	1·57	19·01	—	—
11 „	7·86	0·31	20·22	1·00	19·26	2·39	15·22	0·76	19·16	—	—	—	—	1·50	18·87
16 „	8·18	0·39	20·08	0·97	18·25	1·16	16·20	0·31	20·25	0·76	20·06	—	—	—	—
21 „	8·28	1·08	19·61	2·46	18·98	1·58	17·10	0·54	20·24	—	—	—	—	1·30	18·74
Mean				1·61		1·62									
Set 2. Entire plants, in badly aerated water:															
4 May	1·38	1·18	19·38	1·93	15·99	3·26	14·03	—	—	—	—	1·89	17·83	—	—
9 „	0·25	0·79	19·53	2·44	16·22	1·87	12·98	1·48	17·03	2·13	16·93	—	—	—	—
15 „	0·18	0·25	20·46	1·57	16·10	3·19	16·14	0·77	19·92	1·97	19·44	—	—	—	—
19 „	0·00	0·67	20·32	1·89	17·60	3·04	15·39	1·13	19·54	1·52	19·36	—	—	—	—
23 „	0·00	0·57	19·92	1·40	14·58	2·00	11·88	1·19	18·17	—	—	—	—	3·54	15·51
Mean				1·84		2·67									

(1) to ensure that in the conditions designed to surround the roots with badly aerated water, the entire mass of water should have minimal oxygen concentration;

(2) to find out how far the root oxygen was influenced by the concentration in the leaf bases.

As in the first series of experiments, a layer of peat was placed at the bottom of a bucket of water and the water was covered with a layer of medicinal paraffin in order to prevent contact between the water and air. The water soon became entirely depleted of oxygen, in fact, it became strongly reducing, for it was noticed that when at the end of a Winkler estimation the sample was left open to the air and starch added, it did not turn blue in the course of time as usually happens when the sample is merely deficient in oxygen. Towards the end of the series, however, this was counterbalanced by the activities of some green algae which developed in the surface and caused the water to contain traces of oxygen. The values for dissolved oxygen in the surface layers of the clean well aerated water were much more variable in this second series of experiments than in the first. This may have been partly due to temperature variation but probably more largely to green algae which reached a maximum development about the middle of July. It was considered to be, if anything, an advantage because if the root oxygen was closely dependent on the external medium such a range of variation might be expected to bring out this fact.

Half the plants were left with their leaves intact; the other half had their outer leaves cut short 3 cm. above their base, leaving the rest intact. In some cases only the type B leaves were left, in others both types B and C were left, the intention being to find out how large a part was played by the type C leaves in the aeration of the roots. The plants were divided out into the two contrasted conditions of aeration in the way expressed in the following scheme:

Plants with all leaves intact:

Well aerated water.	5 plants.	Set 3.
Badly aerated water.	5 plants.	Set 4.

Plants with some leaves cut:

Well aerated water.	Type D cut.	2 plants.	Set 5.
	Types C and D cut.	3 plants.	Set 5.
Badly aerated water.	Type D cut.	3 plants.	Set 6.
	Types C and D cut.	2 plants.	Set 6.

The results, given in Table V, are grouped according to the four sets of plants indicated here.

The procedure for obtaining samples was much the same as for the first series, the following being the points of difference:

(1) Most of the plants produced new roots in culture and these were used as well as intact old ones, of which there were not many.

(2) When taking plants out of the badly aerated water, part of the surface was cleared of paraffin with a strip of stiff paper so that the roots of the plants did not become coated with paraffin and so contaminate the G.S.M. Any paraffin adhering to the leaves was washed away in the rapid rinsing under the tap.

(3) When the plants of sets 5 and 6 were taken out of water the cut ends of the leaves were inevitably exposed to the air for a few seconds. It was evident that they were sufficiently clogged with water to prevent any diffusion of air into the air spaces during this short time. If they had remained exposed while the root samples were being obtained, however, diffusion might have had time to occur, and hence with sets 5 and 6 the leaf base samples were taken first and the root samples afterwards.

(4) In cases where the type C leaves had been removed, the "leaf" sample was obtained by cutting off all the type B leaves about 20 cm. above the water surface and extracting the gas from the next 20 cm. or so above this point for all of them together.

(5) Some of the plants had young offshoots and the gas was extracted from the base of the bud by squeezing, as with the stock, in order to see whether the oxygen concentration was close to that of the parent stock.

The results for the second series are shown in Table V, in the same manner as those given by Table IV.

Limitations of the method

(1) In many cases it was not possible to obtain enough gas in the sample to allow two analyses of it to be made. This was so more particularly with the root and stock samples, but happened occasionally also with the leaf bases if these had become waterlogged or if there was only one, as happened sometimes with the type C base samples. Just over half the values shown in Tables IV and V are the mean of two analyses. In a few cases the volume from the roots was so small that very little reliance can be placed on the result. For these the numbers are bracketed. For the remainder, the volume was enough to give one analysis of which the results are trusted within the limits about to be discussed.

(2) For the seventy-two samples which allowed two analyses the

difference between the two results was not more than 0·5 per cent of the total volume analysed (as distinct from 0·5 per cent error on the results) for either carbon dioxide or oxygen, except in six cases. In these the difference was as much as 1 per cent, and two causes of the error are suggested:

(*a*) An error in the actual analysis owing to small bubbles sticking to the sides of the Bonnier and Mangin apparatus. When this happens in a part of the tube which is visible the bubble can be picked up again by the main thread of gas, but where the tube is hidden in the mercury cup the existence of the bubble is unknown and hence the volume of gas is too small. This error has probably occurred in two out of the six cases since the oxygen value was higher in the second analysis than in the first and the carbon dioxide the same or lower.

(*b*) In the other four the oxygen was lower in the second analysis and the carbon dioxide higher. This could be due to an error similar to (*a*), but might also be caused by some kind of contamination of the gas sample in the sampling tube.

It is thus possible that a few of the results for samples only analysed once may be as much as 1 per cent out on the true value. The possible effect of contamination was reduced to a minimum by analysing the small samples as soon as possible. As a matter of fact, the essential parts of the results would still hold even if every analysis were out by this amount.

(3) It may be objected that to immerse plant tissues in such a liquid as the G.S.M. may alter their metabolism so that rapid changes in gas composition are brought about before the gas can be removed from the plant. This is felt to be unlikely in the case of roots and leaf bases where the internal air spaces are large compared to the volume of living tissue, and where only a very short time elapses between putting the plant in the G.S.M. and collecting the sample. That there may be such an effect during longer periods of time has already been mentioned in the case of leaf tissues. On another occasion, during a preliminary experiment, a stock which had been left for about 1½ hours in the G.S.M. gave a much lower oxygen value than was expected from other considerations. Hence for the stock and rhizome, which were sometimes in the G.S.M. for periods up to half an hour, the oxygen values must be considered as minimal.

Results

The main conclusion which will be derived from Tables IV and V is that the oxygen values for the root and stock are closely correlated

Table V. *Results of second series of analysis experiments. Plants marked * had both types D and C leaves cut short.*

Date	Dissolved oxygen in water	Leaves		Roots		Stock		Type D bases		Type C bases		Types D and C bases		Rhizome buds	
		CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2
Set 3. Entire plants, in well aerated water:															
19 June	4·46	0·69	20·31	1·69	17·17	3·76	13·94	0·99	18·78	—	—	—	—	1·19	16·47
29 ,,	5·25	0·65	19·94	1·46	12·40	1·26	18·51	0·80	20·25	1·17	19·33	—	—	—	—
17 July	9·26	1·20	19·75	—	—	2·31	15·30	0·89	19·70	2·65	17·28	—	—	—	—
27 ,,	5·83	—	—	1·88	17·71	1·79	15·58	0·92	19·56	—	—	—	—	2·02	17·88
1 Aug.	3·89	1·05	19·66	1·04	16·23	2·00	12·82	1·12	18·60	1·83	17·39	—	—	—	—
Mean				1·52		2·22		0·94							
Set 4. Entire plants, in badly aerated water:															
26 June	0·00	0·42	19·88	[1·69	5·06]	5·38	0·87	3·78	0·49	—	—	—	—	—	—
9 July	0·00	0·85	19·52	3·28	9·67	3·45	7·31	3·18	12·23	5·75	11·21	—	—	—	—
21 ,,	0·00	—	—	[2·96	1·48]	2·68	12·28	1·34	15·82	—	—	—	—	3·62	12·49
25 ,,	0·00	1·10	19·26	2·58	9·65	3·40	12·20	2·06	15·37	2·26	14·43	—	—	—	—
6 Aug.	0·47	1·06	19·68	1·59	11·18	2·25	11·52	1·73	15·44	2·20	14·27	—	—	—	—
Mean				2·48		3·43		2·42							
Set 5. Plants with some leaves cut, in well aerated water:															
*25 June	3·52	0·58	20·02	0·89	3·52	3·02	1·96	—	—	—	—	3·39	1·80	2·34	2·14
*11 July	7·32	1·34	19·10	1·65	6·86	2·98	3·72	—	—	—	—	2·09	7·81	—	—
*23 ,,	6·04	0·93	18·91	3·79	4·75	2·71	5·76	—	—	—	—	1·97	9·60	—	—
31 ,,	5·24	0·70	19·95	1·86	9·76	6·05	7·35	2·04	13·90	—	—	—	—	—	—
10 Aug.	3·65	0·72	19·80	3·05	11·08	2·41	9·59	1·76	16·40	1·77	16·70	—	—	—	—
Mean				2·25		3·43									
Set 6. Plants with some leaves cut, in badly aerated water:															
*27 June	0·00	1·28	18·46	2·89	0·09	3·87	0·00	—	—	—	—	3·58	1·06	—	—
13 July	0·00	1·28	19·38	2·41	0·56	2·59	0·35	2·61	0·68	3·12	8·87	—	—	—	—
*18 ,,	0·00	1·45	18·18	[2·17	1·45]	2·99	1·15	2·66	0·00	3·33	0·34	—	—	—	—
29 ,,	0·00	0·91	19·23	[0·00	0·00]	5·07	0·65	—	—	—	—	5·21	0·43	—	—
14 Aug.	Trace	0·68	20·13	1·58	0·28	2·07	0·50	1·25	0·31	—	—	—	—	—	—
Mean				2·29		3·32									

with the values found for the submerged bases of the non-growing leaves of the plant. Before dealing with this, however, some of the minor conclusions will be stated.

(1) *Carbon dioxide values.*

The results for percentage carbon dioxide show great variation within each set of plants, which is not surprising considering the variety of factors which affect the observed values and the large size of the probable error compared with the actual percentages measured. Hence, while a few generalizations may be made from the results, not very much weight can be attached to them. The first is that, throughout, the mean value for the stock is higher than that for the roots of the same set of plants. This may be due either to more compact tissues or to the greater ease with which gas could diffuse out of the roots into the surrounding medium; possibly both are true. Another noticeable difference is shown between the mean values for type D bases in sets 3 and 4. This can probably be correlated with a much higher carbon dioxide concentration in the water surrounding the leaves in set 4. No estimations of this were made, but it seems reasonable to assume such a state of affairs when the presence of the peat in the water of set 4 is considered. Lastly, as would be expected, the leaves show a generally lower level of carbon dioxide values than the rest of the plant.

(2) *Oxygen values.*

(*a*) *Leaves.* Throughout the experiments only one case occurs where the oxygen concentration is less than 18 per cent and this was on 1 May, when, as already mentioned, the leaf was taken from the plant towards the end of the experiment so that the result is not really comparable with the others. The latter are fairly uniform and the individual variations do not seem to be explained on either physical or physiological grounds.

It may be mentioned that values of 18 per cent to 20 per cent oxygen were also found for leaves removed at other times of day, in preliminary experiments, and for leaves from plants which had been kept in the dark for a fortnight.

(*b*) *Buds.* In the four cases investigated the oxygen concentration was not widely different from that of the parent stock. This is what would be expected since both stele and cortex of the rhizome are continuous with those of the stock. In set 3, the bud oxygen is definitely higher than that of the stock. This is attributed to the fact

that a much smaller thickness of tissue separates the air spaces of the rhizome from the external medium than in the case of the stock, where in any case the volume of air space is relatively small. Hence the concentration in the rhizome air spaces will be more nearly in equilibrium with the external concentration than will that in the air spaces of the stock.

(*c*) *Roots, stock and leaf bases.* The central part of the results are summarized in Table VI below. This shows the mean oxygen concentrations for each set, excluding those five plants for which no significant root values were obtained. Thus twenty-five plants are involved.

Table VI

Set	Dissolved oxygen in water	Roots	Stock	Leaf bases
1	8·0 High	18·7 High	16·2 High	19·7 High
2	0·4 Low	16·1 High	14·1 High	18·5 High
3	4·8 High	15·7 High	15·2 High	19·3 High
4	0·1 Low	10·2 Medium	10·3 Medium	14·3 High
5	5·2 High	3·5→11·0 Low→Medium	2·0→9·6 Low→Medium	1·8→16·4 Low→High
6	0·0 Low	0·3 Low	0·3 Low	0·7 Low

In calculating the means for the last column, the value for the type D bases has been used wherever these had been analysed separately, that is, for eighteen out of the twenty-five plants. For four experiments (25 June, 11 and 23 July in set 5, and 27 June in set 6) the value used is that for types D and C together and in the remaining three cases (1 and 7 May in set 1, 4 May in set 2) the value is that for bases of all leaves together.

To emphasize the significance of the numbers in Table VI they have been characterized as high, medium, etc.; these adjectives are, of course, relative only. In set 5 there has been such a marked drift in values that to insert the mean of them all would obscure the facts. The reason for the drift is difficult to explain but it serves a useful purpose in showing obviously that the root and stock values follow those of the leaf bases rather than that of the external medium. This is, in fact, the central conclusion to be drawn from Table VI as a whole and is the main result of these analysis experiments. It means that when a gradient is set up between a low concentration sur-

rounding the roots and a high concentration in the type D bases, oxygen can diffuse down to the roots rapidly enough to maintain a concentration there which is probably sufficient for the needs of the root tissues. When these bases do not contain a reasonably high concentration of oxygen, say above 2 per cent, that in the roots falls almost, if not quite, to zero, although there may be roughly atmospheric concentrations in the upper parts of the green leaves.

Most of the "leaf-base" results refer to type D bases and the attempt to differentiate between the parts played by types C and D in the aeration of the roots was not successful because the numbers of plants used were too few. To clear up this point and to confirm the general conclusion a third set of experiments was carried out in the summer of 1936. They were more limited in scope but aimed at greater uniformity and accuracy. The methods used were essentially the same as in the second series: half the plants were kept with their roots in clean water, while the other half were grown in water overlying a layer of peat, the water surface being covered with a layer of medicinal paraffin. Some plants had their type D leaves cut off about 2 cm. above their bases, in other cases both types D and C were treated in this way. Six sets of three plants each were set up as follows:

Plants with all leaves intact	Aerated water	Set 7
	Non-aerated water	Set 8
Plants with type D leaves cut short	Aerated water	Set 9
	Non-aerated water	Set 10
Plants with types D and C leaves cut short	Aerated water	Set 11
	Non-aerated water	Set 12

All but two of the plants for which results are given were put into the culture conditions on 4 May and had thus been there three weeks at the beginning of the analyses. The two exceptions (12 June in set 8 and 17 June in set 12) were used for analysis after they had only been ten days in culture because they had been added later to replace plants which had proved useless for one reason or another.

As before, a sample of the water was taken at the beginning of each experiment for the estimation of dissolved oxygen. The plant was taken out of the water in which it was growing and any mud or paraffin adhering to it was washed off very rapidly under the tap, and then the whole of the base of the plant was immersed in a bowl of G.S.M. All the intact roots were cut off below the surface of the liquid and transferred in a small vessel to another bowl of clean G.S.M. which contained the collecting funnels ready inverted in it.

The gas from the roots was squeezed out with the fingers below one of the funnels. The basal two centimetres of the type D leaves and then of the type C leaves were cut off and similarly transferred to the clean G.S.M. and gas samples of each were obtained. The advantage of this method was that most of the gas contained in the samples never came into contact with any but absolutely clean G.S.M. Since only these three parts of the plants were dealt with, it was possible to obtain all the samples much more quickly than before, i.e. in a time of not more than ten minutes.

The volumes of gas obtained from root samples were larger on the whole than in the earlier experiments, but were not usually large enough to allow of two analyses. The other samples were generally larger, and out of thirty-four analyses which were done in duplicate, thirty-two agreed within 0·5 per cent of the total composition. The remaining ones showed a larger error which could only be attributed to the loss of a bubble, as was discussed earlier.

The results are shown in Table VII, the mean being given where two analyses were carried out.

(1) *Carbon dioxide.* As in the earlier experiments the carbon dioxide concentrations vary greatly within any one set of plants. Except for the very high value given by the first plant of all (26 May in set 7) which is not easy to account for, the results for roots do not appear to vary significantly. Those for type C bases show higher values in set 12, which is what might be expected since the cut end of the leaf base is in contact with the peaty water, and the latter probably has a high carbon dioxide concentration. The type D bases, probably for the same reason, show higher mean values where the leaves are cut short and the water is unaerated.

(2) *Oxygen.* The oxygen values given in Table VII have been plotted in Fig. 4. The chief points to be noticed are:

(*a*) There is a high degree of correlation between the values given by the three parts of any one plant.

(*b*) There is good agreement between all three plants of any one set, and the differences between the sets are clearly shown.

(*c*) The oxygen concentrations are influenced largely by the treatment of the plant and only to a small extent by the oxygen content of the surrounding medium. One may for instance contrast the fact that set 8 shows values as high as set 7 though the mean external oxygen concentration is only 0·48 mg. instead of 3·92 mg., with the fact that set 9, in the same external medium, has quite definitely lower values than set 7. The effect of the medium is shown

Table VII. *Results of third series of analysis experiments*

Set	Condition of plants	Culture medium	Date	Dissolved O_2 in water	Roots CO_2	Roots O_2	Type C bases CO_2	Type C bases O_2	Type D bases CO_2	Type D bases O_2
7	Entire	Aerated	26 May	3·60	7·48	17·57	2·72	18·90	2·82	19·18
			2 June	4·28	1·44	18·18	1·07	18·92	0·84	19·43
			8 ,,	3·88	2·26	15·35	2·13	17·47	1·49	18·72
			Mean	3·92	3·73	17·03	1·97	18·43	1·72	19·11
8	Entire	Not aerated	28 May	0·48	3·09	18·58	1·68	19·70	1·79	19·91
			3 June	0·40	1·77	15·92	2·30	16·56	1·73	15·01
			12 ,,	0·57	1·81	16·68	1·77	18·51	1·16	19·38
			Mean	0·48	2·22	17·06	1·92	18·26	1·56	18·10
9	Type D cut short	Aerated	30 May	3·88	2·66	14·52	2·86	15·89	2·44	16·29
			6 June	4·50	2·26	13·60	3·22	15·14	2·12	16·44
			11 ,,	3·91	1·68	14·62	2·93	15·39	2·15	15·77
			Mean	4·10	2·20	14·25	3·00	15·44	2·23	16·17
10	Type D cut short	Not aerated	1 June	0·08	3·74	13·98	2·41	17·53	3·18	15·49
			8 ,,	0·09	3·62	12·56	3·95	12·93	3·91	10·93
			11 ,,	0·05	2·14	10·18	4·37	10·82	4·43	10·39
			Mean	0·07	3·17	12·24	3·58	13·76	3·84	12·27
11	Types C and D cut short	Aerated	28 May	4·06	3·03	3·43	2·94	0·57	3·22	1·45
			4 June	3·98	1·35	3·82	1·53	3·47	1·30	3·88
			9 ,,	3·69	1·37	3·10	3·21	0·00	2·73	0·77
			Mean	3·91	1·92	3·45	2·56	1·35	2·42	2·03
12	Types C and D cut short	Not aerated	29 May	0·00	3·28	1·67	6·71	0·39	3·54	2·26
			5 June	0·06	1·90	0·82	5·99	0·15	2·94	0·33
			17 ,,	0·18	3·01	1·15	3·81	1·09	3·74	0·49
			Mean	0·08	2·73	1·21	5·50	0·54	3·41	1·03

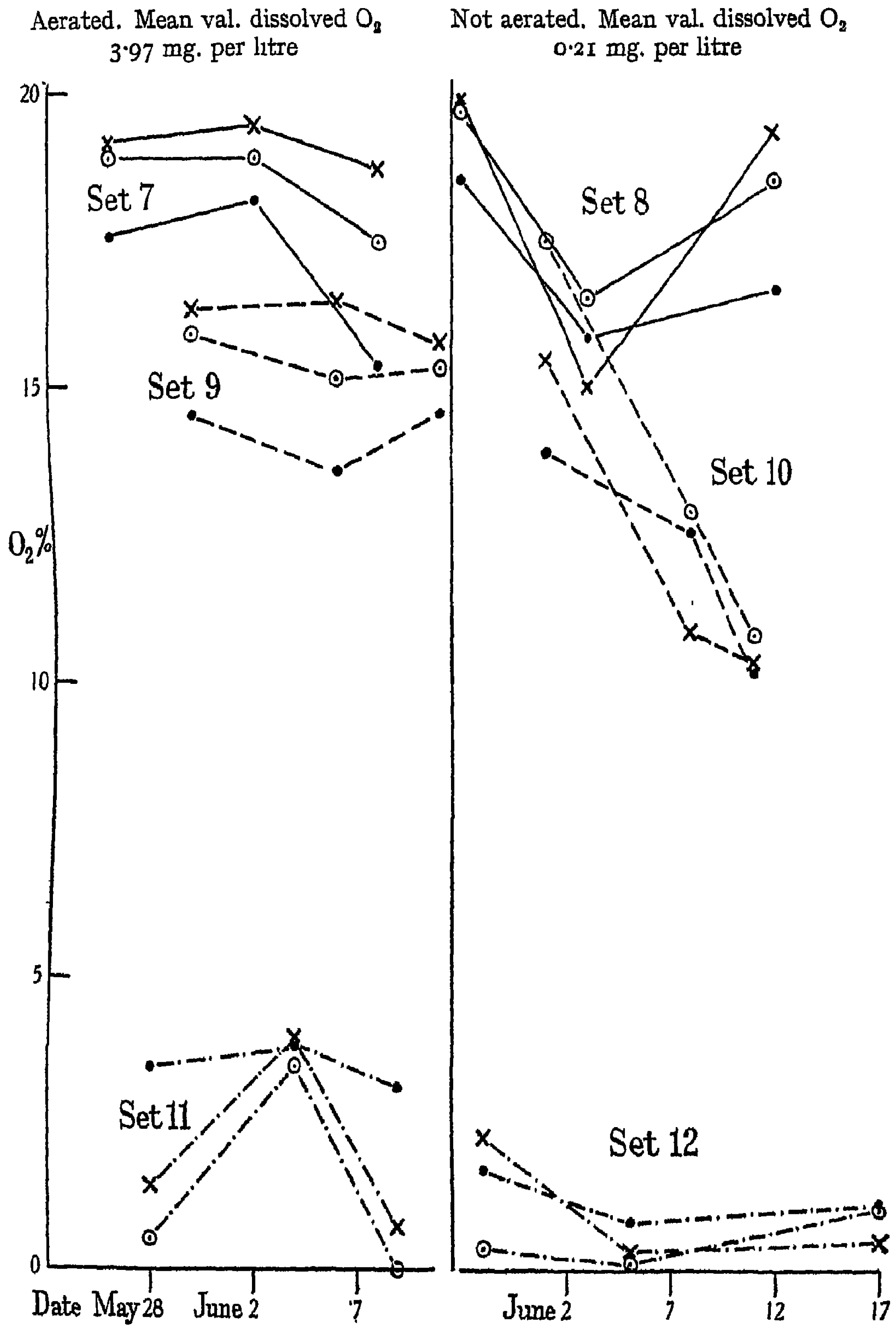

Fig. 4. Oxygen concentrations in third series of analysis experiments. ——— Plants entire; – – – – – Type D leaves cut short; –·–·–·– Types C and D leaves cut short; • Roots; ⊙ Type C bases; × Type D bases.

only by the slightly lower general level in set 12 as compared with set 11, and possibly by the downward drift of values with time in set 10. This may indicate that equilibrium has not been reached, and that the values in set 10 would ultimately be well below those in set 9, but it could equally be mere coincidence, since the number of plants is so small, and the individual variation large enough to prevent one placing any weight on this interpretation.

(*d*) If the type D leaves cannot serve as a channel through which air may diffuse to the submerged parts of the plant, the oxygen concentrations fall, but not to a very large extent. If, however, the type C leaves are also involved, the oxygen values fall to very low levels. This result is exactly parallel, as should be expected, with the porometer results, as may be seen on comparing Fig. 4 with the data for plants R, S, and T in Table III. This agreement indicates the validity of the two methods and strengthens the conclusions drawn from them.

(*e*) The oxygen values for type C bases are lower in set 9 than in set 7, even though the type C leaves are entire in both cases. This is natural since if the diffusion path is narrowed the gradient along the path will become steeper and since the leaf bases are close to the low oxygen end of the gradient for the plant as a whole the steepened gradient will be apparent in the lowered values in the type C bases.

It may be doubted whether the concentrations found in sets 11 and 12 would allow of any growth of new roots. It is noteworthy that though these plants remained alive and their leaves continued to grow in the culture conditions, the leaf lamina that was produced then was of the bright green and mechanically weak type that is always associated with adverse conditions in this species. The same effect was seen in sets 5 and 6 in the second series of analysis experiments.

The great importance of the non-growing leaves in allowing the aeration of the lower parts of the plant makes it possible to suggest why *Cladium Mariscus* usually shows a fairly definite lower limit to the depth in which it can grow. For if too large a proportion of the leaves are submerged they will be liable to rot much more rapidly, and the length of leaf through which the oxygen must diffuse is so much greater that the rate at which it can reach the stock and roots may well be insufficient. The species may be compared with *Phragmites communis* where the growing-point is not long submerged, and the hollow aerial stem is continuous with the rhizome.

This may be one reason why *Phragmites* is usually found colonizing deeper water than *Cladium*.

Summary

The internal air space system of an aquatic species has been investigated with regard to the ease of passage of gas from one part of it to another, in relation particularly to the means by which the submerged parts of the plant are supplied with oxygen. Experiments on mass flow of air and those on diffusion of oxygen according to the concentration gradient gave closely parallel results.

1. Air spaces are present in almost all the tissues of the plant and are linked up into a continuous system.
2. The proportion of air space to cell varies greatly, being as much as 60 per cent in the root. As might be expected the ease of passage of gas through a tissue is correlated with the proportion of air space in the tissue.
3. Gas can pass down to the stock and so to the roots very much more easily by way of the types C and D leaves than by the type B leaves. The meristematic region at the base of the latter only allows gas to pass through it with great difficulty.
4. Provided the bases of the types C and D leaves are present in their normal intact condition it is possible to have oxygen concentrations of 16–18 per cent in the root air spaces though the dissolved oxygen of the surrounding medium is equivalent to an atmospheric concentration of 0–1 per cent.
5. If the type D leaves are blocked up the root oxygen is slightly lowered, ranging from 10 to 15 per cent.
6. If, however, the type C as well as the type D leaves are blocked then the root oxygen falls to very low values even though the external concentration be high. Thus a concentration of 3 or 4 per cent may be found in the roots though that outside may be equivalent to 15 per cent (7 mg. per litre).

From the results summarized above it will be apparent that in the mature plant of *Cladium Mariscus*, the roots growing in unaerated mud are dependent for oxygen supplies upon the bases of leaves already dead (type D) and upon leaves which, though still green, are not growing (type C). The growing green leaves (type B) do not contribute to this gas supply, though at first sight they might have been expected to do so. This may be attributed to the active basal meristems which are almost devoid of air spaces and offer great resistance to the diffusion of gas into the subaerial parts of the plant.

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REVIEWS

Protoplasm. By W. SEIFRIZ. 9×6 in. Pp. x+584 with 179 figs. McGraw-Hill, New York and London, 1936. Price 36*s*.

Prof. Seifriz has investigated a very wide range of natural phenomena, and he here presents the results of his experiments and reading in the simplest possible language. He explains that he is writing for students, not for his professional colleagues in biology; but there can be few readers of any status, who could not find much to interest them in these pages. This results not only from the matter, but also from the manner of its presentation, for Prof. Seifriz has the gift of the happy phrase, and has not been afraid, because he is writing a scientific text book, to use it. For example: "Perhaps we have deified the hydrogen ion", which summarizes accurately enough what many have thought; or again, "In judging the value of criticism, we must distinguish between a purely emotional reaction without experimental work to support it and carefully carried out research. Most of the opposition to Gurwitsch is of the former sort ..." (Opinions may differ about the validity of the rider.)

One of the most striking features of this book is its immense scope, no less in the words of the author than "all those parts of the branches of science which bear upon the physical chemistry of living matter". Moreover, even these generous limits are exceeded unless we place a very loose meaning upon "physical chemistry", for we are given chemistry and physics of all branches. Realizing also that in one sense all biological phenomena are protoplasmic, the author has pursued his theme as far as the acidity of soils, electric potential-gradients in stems, coagulation of blood, and speculation upon the origin of living matter; human disease is a very frequent topic in the later sections.

The first five chapters deal with direct protoplasmic observation, and perhaps the most interesting is the chapter on micro-dissection. Since 1928, when Heilbrunn rather devastatingly criticized certain applications of this technique, an easily accessible statement of the other side of the case has been very desirable. Apart from the fact that Prof. Seifriz is a distinguished exponent, he reveals his enthusiasm with exclamations such as "Many are the possibilities of micrurgy". The success of C. V. Taylor in replacing the micro-nuclei of *Euplotes*, and growing it on to form colonies from the treated individuals (described on p. 63) does make such statements understandable. Indeed, "the ingenuity of these micrurgists seems to have no limit". Prof. Seifriz's attitude towards the usual objection to the method is given in the following paragraph: "The reaction of an amoeba to mechanical irritation and the changes (e.g. thixotropic collapse) which protoplasm undergoes on dissection indicate that micrurgical operations may cause very serious alterations in the behaviour of the organism and in the properties of protoplasm. This is true and critics who have never operated a microdissection needle like to tell about it. 'Your cell isn't normal. How do you know that your results mean anything?' When a research worker in medicine studies the action of the heart of an animal under anaesthesia, he is working with an abnormal organism. When exposed muscles and nerves are studied, they are under abnormal conditions. When animal tissues and plant seedlings are grown in culture solution, the environment is abnormal. When cells are treated with salts, when body fluids are withdrawn and then studied, when living tissues are stained—in general, whenever chemicals are added or instruments applied to organisms and cells—the material is abnormal. In other words, any living object when subjected to experiment is in a more or less abnormal state. This is experimentation and on it does the advancement of biology depend."

It must, of course, be emphasized that the justification of micro-dissection is not a primary object of the book.

Further chapters are devoted to the structure of protoplasm as suggested by direct observations, the ultramicroscope, and X-ray analysis, permeability, dealt with on conventional lines; electrophysiology; and the role of water, salts, and hormones. In dealing with osmosis, adsorption, electrokinetics, radiant energy, the carbohydrates, the proteins, etc., a concise exposition of the general subject is first given, and is followed by its applications "in the living world". The amount of information compressed into these chapters is enormous.

In covering so vast a field with the work of a single pen, it is inevitable that some parts should be better done than others; and it is noticeable that the author is not altogether at ease with questions of pure chemistry. Thus there are rather frequent statements of the following kind, "They" (the sugars) "are reduced by oxidation to their simplest member glucose" (p. 455); "...the utilisation of this sugar (oxidation to lactic acid)..." (p. 456); "The hemicelluloses are incompletely developed forms of cellulose and other carbohydrate materials such as araban, xylan, etc." (p. 459). Neither of the possible meanings of this sentence seems permissible. Further, "we believe that chlorophyll contains something that is responsible for the synthesis of sugar from carbon dioxide and water; we call this something an enzyme and name it chlorophyllase" (p. 508), "These bacteria use sulphur instead of carbon in the synthesis of higher foods" (p. 432); "The action is an exothermic one; i.e. it proceeds without elimination of heat (p. 458)." A further criticism that might be made of the chemical sections concerns the choice of matter; it is impossible for a biologist to enthuse over an account of the carbohydrates, which mentions neither stereoisomerism nor the active sugars.

It would seem that the time has now come when a knowledge of elementary biochemistry may be assumed in the advanced students of biology for whom this book is suitable, and very little would have been lost by the omission of a good deal of chapters 23, 24 and 25. The point is not mentioned out of captiousness, but because it seems a pity that the high price will inevitably place this book out of the reach of the great majority of those students for whom it is specifically intended, and because a severer pruning of the contents would have been one way out of the difficulty. It must, however, be made clear that an immense amount of pabulum has been served up in an appetizing and readily digestible form; it is garnished with excellent photographs and diagrams, and has firmly held the interest of at least one reader.

W. O. JAMES

Morphology of Vascular Plants. Lower Groups (Psilophytales to Filicales). By ARTHUR J. EAMES. 433 pp., 215 figures in text.

"Morphology" has meant so many things that it was good of the author in his first sentences to tell us what his book is about. "Morphology deals with form...external and internal...in thus fundamentally descriptive...it is comparative in its fundamental nature...its purpose is naturally the determination of the origin and relationships of groups of organisms." Anatomy is reduced to the minimum needed in the explanation of stem, leaf and sporangium relations; its detailed treatment we are told "is beyond the scope of this book".

What we have here is a straightforward account of the general form of the sporophyte; and a rather more detailed account of the structure of the gametophyte and young embryo of each living group of the Pteridophytes. The fossils are placed together towards the end of the book and there is a final synthetic chapter.

The phylogenetic and morphological theorising are sufficiently restrained and no one need quarrel with any of the conclusions expressed. Where doubt

exists we are told of it; but the way in which a matter is settled in the text often seems to me to do less than justice to Prof. Eames

Two examples must suffice. The spore-sac of *Psilotum* we are told has been regarded either as a septate sporangium or else as a synangium Arguments are given in support of the "septate sporangium" idea alone and judgement is reached in its favour. Some pages later when the matter is treated as settled it is mentioned in another connexion that the septum has a vascular core—as in the sporangium of no other living plant. Surely this was a strong argument in favour of the synangial view? I am sure that this apparently one-sided manner is due to mere lack of space.

Then in dealing with the Osmundaceae he figures a typical *Todea* fertile pinnule, a typical one of *Osmunda* and some pinnules of *Osmunda* of intermediate character forming a gradual transition between the two genera He says "These transitional leaflets which have both green blade and sporangia show that in this family the...." Well, we are told the *Osmunda* end of the series is primitive but as far as I can see it might just as reasonably have been *Todea*! Nothing in this or the next paragraph gives me any help I can see that the intermediate pinnules show that *Osmunda* has more or less latent in it the potentiality of producing *Todea* pinnules, but surely I want some clue to show me when to regard such abnormalities as "reversions" and when as apparently here to regard them as prophetic? Here the fault if not my stupidity seems to be due to the author's assumption that we share his basic principles of comparative morphology—principles which are not explained and which we must guess from such arguments

The chief lack in this book is not anatomy but physiology. We have it is true plenty of ecological adaptions and some physiological anatomy (of Haberlandt's sort) but there is little discussion of working mechanism. Is it adequate in a book of some 400 pages on the Pteridophytes to say of the action of fern annulus just this: "The function of the annulus at first is merely to open the wall allowing the spores to sift out; when most specialized, it actively disperses them"? Nor is there any suggestion of causal morphology: thus the ordinary "tetrahedral" spore is repeatedly described and figured, but it is never explained how this is formed from the dividing mother cell, still less that this is a minimum surface arrangement for dividing a sphere into four equal parts. Some readers of this book may hold however that Prof Eames did right to exclude "causal anatomy" which is often nonsense and when sound is felt to give the enemy (plant physiologist) a dangerous footing in the morphologist's territory.

It must not be supposed from these few points of adverse criticism that this is not a valuable book: it is Nor do all conclusions seem dictated to us; in many places, especially in the final chapter, the student will gather Prof. Eames' opinion while realizing that those who think otherwise may also be reasonable and even right. Personally, I have learnt a great deal about the Pteridophytes, particularly about their gametophytes, for which I am grateful. The carefully selected bibliography at the end of each chapter is sure also to be useful.

TOM H. HARRIS

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THE ACTION OF AMYLASES IN RELATION TO THE STRUCTURE OF STARCH AND ITS METABOLISM IN THE PLANT. PARTS I-III

BY CHARLES S. HANES

From the Low Temperature Research Station, Cambridge

(With 5 figures in the text)

INTRODUCTION

ONE of the most striking features of the biochemical activity of the plant cell is the versatility it displays in promoting interconversion of carbohydrate substances, a characteristic which is reflected in the wide variety of compounds of this group occurring in the plant body and the rapidity with which they may be transformed. Many carbohydrates of great importance are known exclusively as products of plant metabolism and, not only has it been impossible to effect their synthesis under artificial conditions, but in many cases no clue has been discovered as to the manner of their formation in the living plant.

The polysaccharides, as a group, provide a most interesting and important example. Polysaccharides are presumably elaborated from simple sugars in the plant, but no insight has been gained concerning the mechanism by which the synthesis is effected, nor the intermediaries which may be involved. The process remains an obscure and inimitable endowment of the living cell.

The traditional interest of the plant physiologist in the amylase group of enzymes is clearly related to this fundamental problem of polysaccharide metabolism. Starch is the most familiar example of a polysaccharide which, in contrast to the cell-wall polysaccharides, is subject to rapid reconversion into sugars in the living cell and so constitutes a readily available reserve of simple sugars. The problem of the metabolism of starch in the plant accordingly presents two aspects, synthesis and degradation, and it has long been believed that

information concerning the latter process is likely to elucidate the nature of the former.

Starch-splitting enzymes have been demonstrated to occur in many different species and organs of plants. There is a general conviction that they play an essential metabolic role, and the view is widely accepted that the amylase system constitutes the normal cellular mechanism by which starch is converted into sugar in the living plant. It is often assumed further that amylase, in addition to promoting the hydrolytic reaction, may under appropriate conditions facilitate the reverse process, and so form at least a part of the still mysterious mechanism of starch synthesis. Up to the present, however, there has been no authentic demonstration that the normal hydrolytic action of amylases is reversible at any stage in the breakdown of starch or under any conditions which have so far been investigated.

It need scarcely be emphasized that the familiar expression "Starch $\rightleftharpoons$ Sugar (catalysed by amylase)" summarizes a highly speculative hypothesis. There will be occasion later to consider the complexities, both chemical and biological, which characterize the processes which are represented so simply in this expression.

The formulation of views concerning amylase action in the living cell must be based largely on conceptions of the action *in vitro* of extracted amylase preparations, and the major part of the present review will be occupied in considering the information which is available in this field.

It is evident that the mode of action of an enzyme can be deduced only from a clear conception of the chemical nature of the substrate, and of the products of its action, together with a knowledge of the order in which the chemical events occur. The relatively limited progress which has been made in elucidating the action of amylases is traceable to the uncertainty which has prevailed in regard to these fundamental issues. Thus, while various criteria have been available by which the progress of enzymic starch degradation may be followed accurately, it has not been possible to interpret these measurable changes in terms of the precise chemical events which underlie them.

With the emergence during recent years of the definite conception of the molecular constitution of starch, which has resulted in the main from the purely chemical investigations of Haworth, Hirst and their associates in the Birmingham laboratory, rapid progress seems now to be expected in solving these basic aspects of the starch-amylase

problem. The striking advances which have been made encourage the hope that it will be possible in the near future to formulate precisely the specific enzyme-substrate relationships which characterize the different recognizable types of amylase.

Already it appears that a consideration of this chemical view of starch structure, in relation to the established facts concerning starch degradation by amylases, results in an appreciable clarification of the general subject, and, of equal importance, it serves to throw into prominence a number of specific problems, the solution of which appears to be of critical importance in these interrelated fields.

PART I. THE CHEMICAL CONSTITUTION OF STARCH

THE PROBLEM OF POLYSACCHARIDE CONSTITUTION

The chemical constitution of polysaccharides has presented to the chemist a problem of exceptional difficulty and, until recently, even the basic structural plan of the polysaccharide molecule has been a subject of controversy. Two opposing views on this question have been much debated.

(*a*) *The traditional saccharide chain hypothesis.* On the one hand the polysaccharide molecule has been regarded, according to the view held by Emil Fischer, as consisting of a chain structure made up of numerous monosaccharide units joined together by the ordinary valency forces. Thus the polysaccharides were conceived to represent high analogues of the simpler saccharides (such as di-, tri- and tetrasaccharides) in which the units are joined by glucosidic bonds. As will be seen later, this traditional view of polysaccharide constitution has received convincing support within recent years.

(*b*) *The low molecular "elementary unit" hypothesis.* An entirely different conception, first advocated rather more than a decade ago, was supported by a certain group of investigators and came into considerable prominence. The basic idea of this hypothesis was that the true molecules of polysaccharides are relatively simple "elementary units" which were conceived to be anhydrosaccharides of crystalloidal dimensions. These low molecular "elementary units" which would be represented by the same empirical formula as the polysaccharide itself—$(C_6H_{10}O_5)_n$ in the case of starch—were believed to possess, as a result of structural peculiarities, the property of associating together to form large aggregate molecules of colloidal dimensions. The association into aggregates, and hence the colloidal

nature of polysaccharides, was considered not to involve the usual interatomic valency forces, but was attributed to some form of intermolecular attraction (residual, auxiliary, or co-ordinate valencies).

It would constitute a digression to trace in detail the rise and decline of the "elementary unit" hypothesis, but certain general comments are clearly justified. Much energy was expended in the search for the respective units of cellulose, starch and inulin, and rival proposals were made as to their nature. For starch, the following range of compounds was suggested as representing the "elementary unit":

Glucosan (anhydride of glucose), Bergman & Knehe (1927).
Maltosan (anhydride of maltose), Karrer & Nägeli (1921).
Dihexosan (for the amylose fraction) }
Trihexosan (for the amylopectin fraction) } Pringsheim (1924).
Anhydride of a hexasaccharide, Ling & Nanji (1925), Irvine (1928).

It may be stated that, while certain of these views were based on theoretical reasoning which must now be regarded as faulty (for example, the hypothesis of Karrer and of Irvine who suggested anhydro-maltose and anhydro-hexasaccharide units, respectively), the remaining proposals arose from erroneous conclusions as to the molecular size of substituted derivatives of polysaccharides and their degradation products.

The misleading conception of the essentially low molecular character of polysaccharides rested primarily upon cryoscopic determinations of the molecular weight of acetylated derivatives dissolved in organic solvents. It is now generally recognized that, when applied to such substances, the classical method may yield grossly misleading results; the depression of the freezing-point may be many times greater than that expected from the actual molecular size of the product. It is not clearly established whether this anomalous effect is due to the adherence or occlusion of small amounts of the reagents used in the substitution process, or whether some more obscure factor is at work, but the unreliability of the method has been demonstrated in a number of cases.

The example which has been most investigated is the so-called "biosan acetate" which was held by Hess (e.g. Hess & Friese, 1926) to be the acetyl derivative of the "elementary unit" of cellulose. This he considered to be an anhydro-disaccharide. On mild acetolysis, cellulose was converted into a product of low reducing power which

appeared to have the molecular weight of an acetylated disaccharide as judged by the lowering of the freezing-point in acetic acid solution. While this observation has not been disputed,[1] subsequent investigations have established unequivocally that the "biosan acetate" of Hess consists in reality of a mixture of acetylated cellodextrins of relatively long chain length (Mark & Meyer, 1928; Freudenberg *et al.* 1929; Haworth, Hirst & Ant-Wuornen, 1932).

Bergman's "glucosan" unit for starch affords an exactly parallel case. The evidence in favour of this view consisted in the observation that acetylated amylose, when examined cryoscopically, appeared to have the molecular weight of acetylated anhydro-glucose. Here again it has been clearly shown that the product in question is complex in character, as will become evident in later sections (cf. pp. 112, 121).

The rapid extension of the "elementary unit" hypothesis must be attributed to the peculiar difficulties which have been encountered in applying the classical methods of cryoscopy to substituted polysaccharide products: such observations, without adequate supporting evidence, can no longer be regarded as convincing. The nature of the products designated by Pringsheim and others as dihexosan and trihexosan must, on these grounds, be regarded as an open question.

It is now almost universally accepted that polysaccharide molecules consist of chain structures containing many hexose units joined together by glucosidic bonds. At the same time, as will become clear, a phenomenon of reversible aggregation of the chemical units is clearly discernible in the case of starch. This, however, is an effect of an entirely different order from that conceived in the "elementary unit" hypothesis. There is no doubt that the chemical units of polysaccharides are themselves of at least low colloidal dimensions.

The considerable clarification of views on the structure of polysaccharides during the past five years has resulted principally from the extension of the methylation method to this group of substances. Owing to the extreme importance of this development to the main subject under consideration, it seems desirable to describe the more recent investigations on starch structure in sufficient detail to permit the botanical worker to appreciate the experimental evidence upon which the conclusions have been based.

[1] The freezing-point depression in certain solvents indicates a high molecular weight of the order of 3000 (cf. Haworth, Hirst & Ant-Wuorinen, 1932).

THE SACCHARIDE CHAIN STRUCTURE OF STARCH

The conception of starch structure proposed by Haworth, Hirst and their collaborators has been elaborated in the course of some seven papers (*J. chem. Soc.* 1928–35). While the principal developments have emanated from these investigations, other workers have contributed important evidence along lines which will be considered in the subsequent sections.

It should be mentioned that, although attention will be confined almost exclusively to progress which has been made in the starch problem, the application of similar chemical methods to other polysaccharides has resulted in comparable theoretical developments, and in certain cases, additional means, so far inapplicable in the case of starch, have been available for verifying the conclusions. An important example is the harmonization of the conception of the molecular structure of cellulose, derived from these purely chemical investigations, with that deduced from the X-ray diffraction pattern.

The Haworth-Hirst model of the starch molecule

According to the view which has now gained wide acceptance, the starch molecule is regarded as consisting of a chain structure made up of α-glucose units joined together by glucosidic linkages. The glucose units are present in the normal or glucopyranose form in which the first and fifth carbon atoms take part in the formation of the internal oxygen ring (Fig. 1). The reducing group (C_1) of each glucose unit is joined with the fourth carbon atom (C_4) of the succeeding unit through sharing an oxygen atom. The glucosidic linkages of the chain structure are accordingly of the α (1–4) type.

In Fig. 1 are set out the formulae of α-glucopyranose, α-maltose and a fragment of the chain structure which will be referred to as the Haworth-Hirst model of starch.

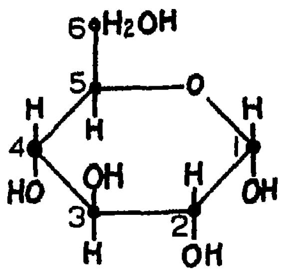
It will be seen that any adjacent pair of hexose units of the structure proposed for starch presents the configuration peculiar to the disaccharide maltose, as established by Haworth & Peat (1926).

The starch molecule is considered by the Birmingham workers to consist of a terminated open chain of this type containing 24–30 glucose units.

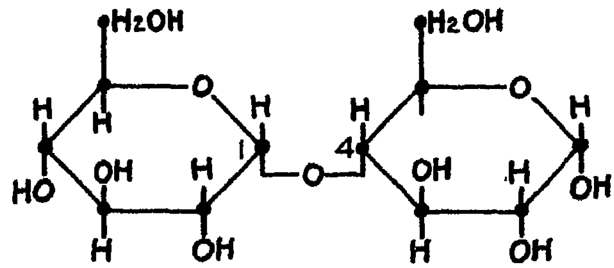
At this point, the view which has arisen concerning the molecular constitution of cellulose should be mentioned. While the starch molecule consists of a chain of recurrent α-glucopyranose units joined through carbon atoms 1 and 4, the molecule of cellulose

consists of a chain of β-glucopyranose units similarly joined, as shown in Fig. 2.

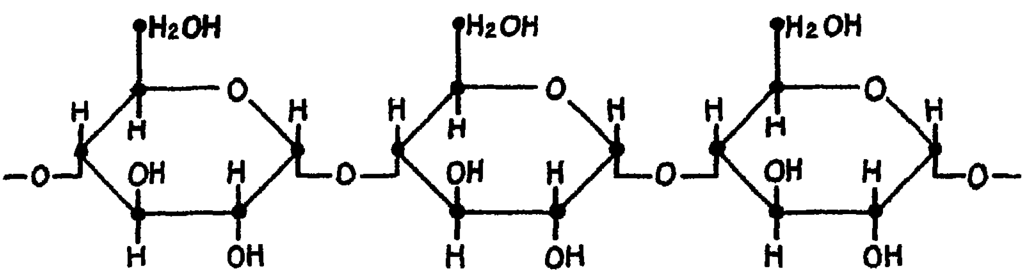
An additional point of difference lies in the number of hexose units comprising their respective chain molecules; the chemical unit



α-Glucopyranose

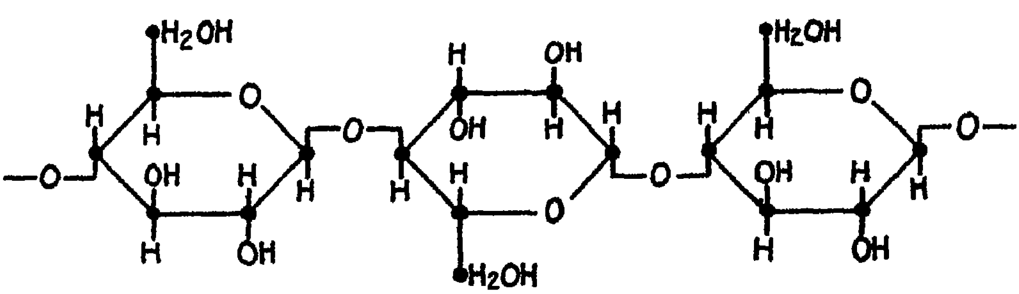


α-Maltose



α-linked glucose chain (starch)

Fig. 1. Perspective formulae. The carbon atoms of glucopyranose are numbered 1–6. At C_1 (the reducing and mutarotating group) the H and OH appear in the α-configuration.



β-linked glucose chain (cellulose)

Fig. 2.

of cellulose is thought to contain about 200 hexose units (about eight times the number present in starch). It is a remarkable fact that, so far as is now known, the differences in the properties of starch and cellulose are to be attributed to this stereochemical dissimilarity, the glucopyranose units being in the α-form in starch and the β-form in cellulose.

It should be pointed out that, whereas the chain structures of both starch and cellulose have been represented above in a strictly linear form, a study of three-dimensional models reveals the fact that only the structure proposed for cellulose would approximate to this rectilinear arrangement. The starch chain structure, which it will be noticed is asymmetrical, probably exists as a spiral. This difference in chain conformation has been emphasized in connexion with the difference in physical properties of the two substances (Hirst *et al.*, 1932; Freudenberg, 1931).

We shall now consider the nature of the evidence upon which this rather detailed picture of the chemical structure of starch has been based. Certain preliminary remarks on the methylation method will facilitate the presentation of the argument.

The method of methylation

Methylation, which consists in the substitution of hydroxyl groups (OH) by methoxyl groups (OCH_3), has come to be one of the most important means available for elucidating molecular structure in the sugar group. This is due primarily to the fact that the substitution can be effected without inducing stereochemical changes, such as Walden inversion, or alterations in ring structure, to which sugars are liable. Moreover, methoxyl groups once introduced have not so far been known to migrate from one carbon atom to another.

The positions occupied by free hydroxyl groups in the original molecule are thus blocked by the firmly fixed methoxyls of the methylated derivative, and such derivatives may be subjected to relatively drastic chemical treatment, such as acid hydrolysis, without removing or displacing the substituted groups. Furthermore, many of the methylated monosaccharides may be separated by fractional distillation *in vacuo* and identified in respect to both the number and position of the substituted methoxyl groups.

It will be seen, therefore, that the method of methylation offers the possibility of establishing the positions of free hydroxyl groups in the monosaccharide units of more complex molecules.

Finally, a distinction should be drawn between the true methyl ethers discussed above and the relatively labile methyl glucosides which are formed, for example, when methyl alcohol condenses with the aldehydic carbon atom of glucose.

The methylation of starch

The starting-point of constitutional studies on starch is the established fact that starch is converted quantitatively into *d*-glucose by acid hydrolysis. The possibility of the presence of more than mere traces of other monosaccharides is precluded.

The first definite suggestion, as to the particular form of glucose which exists in starch, was gained by Haworth, Hirst & Webb (1928), who, as a result of improved technique, were able for the first time to apply satisfactorily the methylation method to the problem.

The methylation of starch had been previously attempted by other investigators with only partial success. Thus, although fully methylated products (containing three OCH_3 groups per glucose unit) had been described, the yields obtained were so small and the procedure for achieving complete methylation so drastic and prolonged, that it seemed doubtful whether the products were, in the first place, representative of the whole of the starch substance and whether they could, in the second place, be considered to be genuine derivatives.

The successful methylation by Haworth and his collaborators depended upon the development of the following procedure, which, with modifications, has been used in subsequent investigations:

(1) Starch was first brought into a finely divided state by its precipitation with alcohol from hot water suspension (starch paste).

(2) The product was acetylated under gentle conditions by treatment with glacial acetic acid and acetic anhydride in the presence of traces of chlorine and sulphur dioxide as catalysts. (On removal of the acetyl groups the original starch could be recovered unchanged.)

(3) The triacetyl starch was then subjected to treatment in which simultaneous de-acetylation and methylation took place. This was achieved by treatment in acetone solution with methyl sulphate and soda.

The transformation in each case proceeded smoothly and gave products in high yield. Thus triacetyl starch corresponding to 96 per cent of the original substance was obtained, and the fully methylated derivative was representative of at least 85 per cent of the original starch.

Danger of degradative and other changes having occurred during the substitution process is minimized by the short duration and the extreme mildness of the treatments involved, and there is no doubt that the methylated products obtained represent genuine derivatives.

The final products could not normally be separated into fractions having dissimilar chemical properties, although it was demonstrated (Hirst *et al.*, 1932) in exceptional cases (where degradation was suspected as a result of faulty control of the conditions) that the presence of degradation products was easily detected and that such products could be completely eliminated by fractionation in organic solvents.

The products of hydrolysis of methylated starch

The acid hydrolysis of such indisputably genuine specimens of methylated starch was next investigated and in this way the individual glucose units, containing unchanged their substituted methoxyl groups, became available for study.

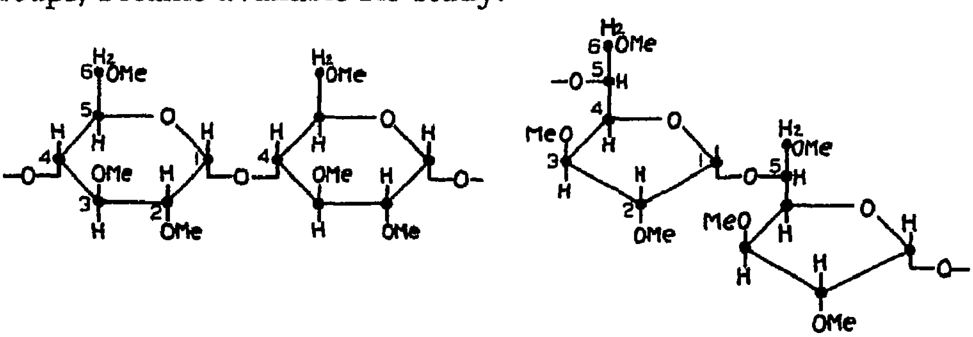


Fig. 3. Alternative structures which would yield 2 : 3 · 6-trimethyl glucose.

An analysis of such hydrolytic products showed the presence in high yield (at least 85 per cent) of 2 : 3 : 6-trimethyl glucose and in addition small amounts of 2 : 3 : 4 : 6-tetramethyl glucose (to be discussed later, p. 112).

These observations established that the glucose units as they pre-exist in the starch molecule have free hydroxyl groups associated with carbon atoms 2, 3 and 6, and, accordingly, that these particular C atoms were taking no part in the formation of either glucosidic linkages between glucose units, or the oxygen bridge which completes the ring structure of each glucose residue.

That C_1, the aldehydic group, is engaged in glucosidic union is clear from the non-reducing character of starch. The observed facts could thus be accommodated by either of the following formulations:

(1) that the glucose units in starch exist in the glucopyranose form (with internal oxygen ring between C_1 and C_5) and that the units are joined by glucosidic bonds between positions 1 and 4; or

(2) that the units are of the glucofuranose, or "active glucose" configuration (with oxygen ring between C_1 and C_4), and are joined by glucosidic bonds between positions 1 and 5.

According to either of these conceptions, as will be clear from Fig. 3, the structure after methylation and hydrolysis would be expected to yield 2 : 3 : 6-trimethyl glucopyranose.[1] It was conceivable that units presenting both these configurations were present in starch.

For a number of reasons, however, the first interpretation alone seemed tenable. Of these, the relationship between starch and maltose was perhaps the most impelling evidence in favour of a chain structure of α-glucopyranose units linked between positions 1 and 4, as in maltose. Subsequent investigations have served to establish the reality of this relationship.

The chemical unit or macromolecule of starch

Accepting for the moment the conception that starch consists basically of a chain of α-glucopyranose units, we may consider certain broader problems of its molecular structure. The question naturally arises as to the form in which the chains exist, whether, for example,

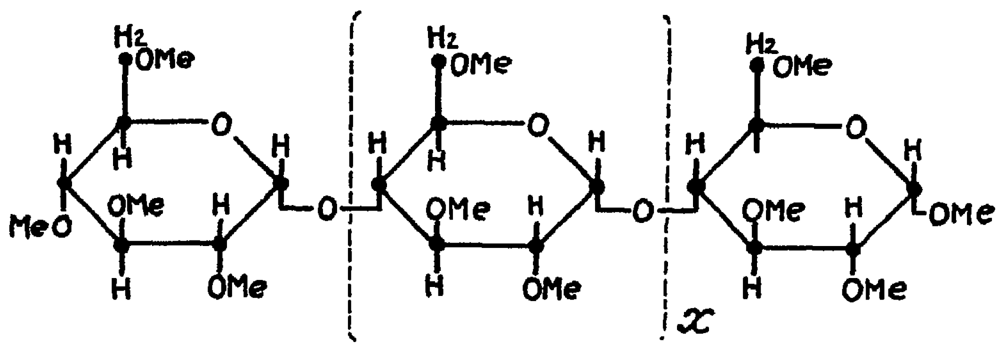


Fig. 4 Methylated starch. Yielding on hydrolysis: 2:3:4:6-tetramethyl glucose, 2:3:6-trimethyl glucose; 2:3:6-trimethyl glucose + methyl alcohol.

they are open chains with free ends, or great endless loops, or possibly some combination of these plans such as a closed loop having one or more attached side chains. Related to these questions is the fundamental issue as to whether starch may be considered to possess a true chemical molecule or whether it consists of heterogeneous assemblages of the basic structural chains.

The Birmingham workers have concluded that a true chemical unit of starch does exist and the view has been advanced that it consists of an open chain containing 24–30 glucopyranose units. The chemical unit so conceived would possess a molecular weight of about 5000. Such a molecular structure, being of colloidal dimensions, is preferably referred to as a macromolecule to distinguish it from "crystalloidal" molecules.

[1] 2 : 3 : 6-trimethyl glucofuranose would revert to the pyranose form on liberation from the chain structure.

The evidence for this view has resulted from the development in the Birmingham laboratory of a purely chemical method for the estimation of chain length (Haworth & Percival, 1932). This procedure, which represents theoretically the simplest means available for establishing the length of open chain structures built up of recurrent units, is based on the determination of the frequency of occurrence of terminal units. It is referred to as the method of end-group assay.

It will be evident that if the chain structure previously set out as the Haworth-Hirst model (p. 106) exists as a terminated straight chain, the terminal glucopyranose unit at the non-aldehydic end will differ from the other terminal unit and from the intermediate chain units in having four non-glucosidyl hydroxyl groupings available for methylation. The fully methylated structure, represented in Fig. 4, will consist of the two terminal units joined by a number of intermediate members.

On hydrolysis, the non-aldehydic end unit will yield 2 : 3 : 4 : 6-tetramethyl glucopyranose, whereas the intermediate units and the aldehydic end unit will yield 2 : 3 : 6-trimethyl glucopyranose.

It was demonstrated that small amounts of 2 : 3 : 4 : 6-tetramethyl glucopyranose are liberated on the hydrolysis of methylated starch specimens. Moreover, an analytical method was perfected (Haworth & Machemer, 1932) for the quantitative determination of this derivative in the presence of the preponderating excess of 2 : 3 : 6-trimethyl glucopyranose which is liberated at the same time.

In practice, the methylated specimen is subjected to acid hydrolysis and the liberated components are converted into the corresponding methyl glucosides. The separation and determination of the tetramethyl methyl glucopyranoside is then accomplished by fractional distillation *in vacuo* and weighing. From control experiments on artificial mixtures of the glucose derivatives in question it would appear that, after correcting for a small loss in distillation (approximately 10 per cent), the values for the yield of tetramethyl glucose are correct to within 5 per cent.

This method of end-group assay has now been applied to a considerable range of polysaccharide products, including starch, cellulose, inulin and a large number of degradation products. We shall now consider only the results obtained in examinations of starch and starch dextrins.

It will be evident that the presence of 2 : 3 : 4 : 6-tetramethyl glucose amongst the hydrolytic products of methylated starch indicates the existence of free ends of the chain structure and, at the same

time, the nature of the terminal unit, while a knowledge of the amount present established the frequency of the occurrence of this terminal unit and so makes possible the estimation of chain length.

The most striking fact which has emerged is that the various methylated starches examined show a remarkable uniformity in the percentage occurrence of the terminal 2:3:4:6-tetramethyl glucose. In Table I are given examples of the published analyses.

TABLE I. *The chain length of starch by end-group assay*

Source of starch specimen	Frequency of occurrence of tetramethyl glucose 1 unit in
Potato starch, separated into amylose and amylopectin by the method of Ling & Nanji (p. 124)	
Amylose	25*
Amylopectin (viscous specimen)	24*
Amylopectin (mobile specimen)	24*
Potato starch (precipitated from "paste" by alcohol)	25†
,, (after conversion into "simplified amylose" or soluble starch, cf p. 126)	26†
Starch from waxy maize (precipitated from "paste" by alcohol)	26–29‡

* Hirst, Plant & Wilkinson (1932) † Baird, Haworth & Hirst (1935). ‡ Haworth, Hirst & Woolgar (1935).

In all cases the yield of tetramethyl glucose indicates the presence of 1 unit of this derivative for every 24–30 glucose units. Accordingly, on the assumption that the molecule is an unbranched chain structure, these figures indicate a chain length of 24–30 glucose units.

In addition to the starch specimens listed in Table I, a preliminary announcement has been made (Haworth, 1935) that similar values have been obtained for the starch of ordinary maize.

It is desirable to emphasize that these different specimens of starch, which are indistinguishable by the end-group assay method, are examples of widely differing varieties (and fractions) of starch. Thus, the starch of waxy maize is abnormal in that it gives a red-violet coloration with iodine, and contains an abnormally high proportion of the readily soluble amylose fraction. Furthermore, the amylose and amylopectin fractions of potato starch, separated by the freezing-out method of Ling & Nanji (1925), gave identical values of chain length, in spite of a profound difference in *physical* properties. The amylopectin fraction, whether in the free condition or in the form of its acetyl- and methyl-derivatives, was less soluble and considerably more viscous than the amylose portion (cf. p. 124), and, presumably as a result of its physical state, greater resistance was encountered in acetylating and methylating this fraction.

These results appear to establish the existence of a well-defined chemical unit of starch consisting of a terminated chain of 24–30 glucose units.

Whether or not this will be found to be true of all starch varieties is a question for future investigation. For the different specimens so far examined, the evidence points to the existence of a true molecule possessing a molecular weight of about 5000, and the important fact has been established that the amylose and amylopectin fractions are not distinguishable in this respect.

The starch dextrins

A number of starch dextrins have now been investigated by the new methods, and the important fact has been established that the dextrins represent fragments of the basic chain structure of varying length. Observations on these degradation products, moreover, have been emphasized as lending support to the view that the macromolecule of starch consists of a terminated open chain structure.

The isolation of the terminal tetramethyl glucose unit from methylated starch does not of itself establish the simple open chain character of the structure, since this might be explained on the basis of a loop structure with one or more side chains attached to it, the terminal units of such side chains being responsible for the production of tetramethyl glucose. It has been pointed out (Haworth, 1935; Haworth, Hirst & Plant, 1935) that if such a conception were adopted, the products formed during the graded breakdown of starch would be expected to exhibit no regular and progressively increasing values for the end-group. It might be possible in this event to isolate a dextrin giving the same end-group value as starch itself. Such has never been found the case; with progressive degradation the products show progressively increasing proportions of tetramethyl glucose units amongst the hydrolysis products of the methylated derivatives.[1]

Chain length determinations by end-group assay have been reported for a number of starch degradation products isolated in a condition approaching chemical homogeneity (Freudenberg *et al.*,

[1] If side chains exist in the structure, the glucose units to which they are attached would be expected to yield dimethyl glucose on methylation and subsequent hydrolysis. This glucose derivative was obtained in small amounts by Hirst *et al.* (1932) from methylated amylose and amylopectin. The production of dimethyl glucose may, of course, be merely the result of failure to achieve absolutely complete methylation, but it may reflect the participation of certain OH groups in other linkages, e.g. those concerned in molecular aggregation (cf. suggestion in regard to xylan, p. 129).

1932; Haworth, Hirst & Plant, 1935; Haworth, Hirst & Waine, 1935). The values indicate that these different dextrins contained 3, 4, 8, 12 and 16–17 glucose units, respectively. A preliminary announcement of the examination of other dextrins (9, 7 and 5 units, respectively) has also been made (Haworth, 1935). These products will now be considered.

Freudenberg, Friedrich & Bumann (1932) separated from the products of mild acetolysis[1] of starch derivatives of saccharides containing 2, 3 and 4 glucose units, respectively. This was accomplished by fully methylating the acetylated breakdown products and converting the mixed methyl derivatives into the corresponding methyl glucosides. The glucosides were then separated by fractional distillation *in vacuo*.[2] The constitutions were established by three independent methods: molecular weight determination; the proportions of tetra- and trimethyl glucose given on hydrolysis (i.e. the end-group assay); and finally the percentage of glucosidic methoxyl groups as distinct from ethereal methoxyl groups. The analyses leave no doubt that these products consist of open chain saccharides, the di-, tri- and tetrasaccharide of the starch series.

Dextrins containing 8 and 12 glucose units, described by Haworth, Hirst & Plant (1935), were isolated from the mixed degradation products formed by heating starch in glycerin. The mixture was acetylated and the separations were made by fractional precipitation in various proportions of chloroform, ether, and petrol ether. The dextrin acetates were then methylated and the methyl derivatives, after further fractionation, were examined by the end-group assay method. Both dextrins displayed a red iodine coloration.

We come now to a dextrin for which a chain length of 16–17 units was indicated by the end-group assay method (Haworth, Hirst & Waine, 1935). This product, which has long been known as Wijsman's erythrogranulose or Baker's α-amylodextrin, is of considerable significance from the point of view of amylase action, in which connexion it will be discussed subsequently (pp. 138 and 139). It suffices at this point to state that by the action of the saccharogenic (β) amylase of barley approximately 60–65 per cent of the starch substance is converted into maltose and there remains a residue representing 35–40 per cent of the starch substance, which is resistant to further attack by this amylase. This residual material constitutes the product in question, for which a chain length of 16–17 units has been reported.

[1] Degradation and simultaneous acetylation in the presence of sulphuric acid and acetic anhydride at moderate temperatures, e.g. 30°.

[2] The principle of "molecular distillation" was used, the distance to be traversed in the gaseous state being reduced to a minimum.

On the view that α-amylodextrin represents a fragment of the original starch molecule, for which there is considerable support, its chain length would be, at the most, about 40 per cent that of undegraded starch. Taking the highest reported value for starch (30 glucose units), it would follow that the α-amylodextrin molecule contains only 12 glucose units.

The reported value of 16–17 units indicated by end-group assay accordingly seems significantly high, and the discrepancy introduces considerations of some importance. On the assumption that starch is made up of *homogeneous* macromolecules containing 30 glucose units, it is clearly impossible that a *homogeneous* degradation of these molecules to the extent of 60 per cent breakdown could result in a residue composed of molecules containing 16–17 units. The acceptance of this value would therefore cast doubt on one or the other of the following features of the general hypothesis: (1) that the macromolecules of starch are homogeneous, (2) that they contain no more than 30 glucose units. In view of the fact that the reported value of 16–17 units for α-amylodextrin appears to rest upon a single determination of the yield of tetramethyl glucose from the methylated dextrin, emphasis of this apparent discrepancy should perhaps await further investigation.

There will be occasion to discuss later other properties of α-amylodextrin, in particular its optical rotation (cf. pp. 122 and 134), and the fact that it exemplifies a class of starch dextrin which displays to a peculiar degree certain properties exhibited by undegraded

[(*Note added* 11 *March.*) The writer is indebted to Professor Haworth for permission to mention the results of more recent experiments on α-amylodextrin. New determinations by end-group assay have revealed a chain length of 12 units for a number of different specimens of this dextrin (Haworth, Hirst, Kitchen, & Peat, 1937, in the press).

The theoretical importance of these observations will be clear from the comments which have been made above. There is now no doubt that α-amylodextrin represents a residual fragment of the original molecular chain structure; the establishment of its chain length as 12 units suggests, moreover, that of the values ranging from 24–30 units observed for starch itself, the higher values are probably more nearly correct.

An alternative view that α-amylodextrin represents a pre-formed starch component will be discussed in connexion with amylase action; this view appears entirely untenable.]

starch. The dextrin exists in an aggregated state, retrograding in solution, and gives a blue-violet colour with iodine.

The main conclusion to be drawn from the recent investigations on starch dextrins is unaffected by the uncertainty regarding the chain length of α-amylodextrin. The observations indicate clearly that these degradation products represent fragments of varying length of the starch chain structure. As will be seen later (p. 121), the optical rotations of these products lend strong support for the view that they represent terminated open chains containing exclusively α-glucopyranose units, linked as in maltose.

An important aspect of the newer work on dextrins lies in the development of methods for their isolation in a state of at least approximate chemical homogeneity. In general, the procedure involves acetylation of the crude products and fractionation of the acetates in organic solvents. The acetate fractions may then be deacetated to yield the free dextrins, or simultaneously de-acetylated and methylated. The methylated derivatives may be further fractionated and the different portions examined by end-group assay, yielding information concerning the range of chain length characterizing the component dextrins.

Although the existing information concerning starch dextrins is by no means exhaustive, the way is opened for more detailed investigations in the future.

The relationship between maltose and starch

At the time the Haworth-Hirst model was first proposed, the pre-existence of maltose in starch had not been conclusively demonstrated. Thus, it had been frequently suggested that the production of large yields of maltose during the enzymic degradation of starch might be the result of a process of reversion-synthesis, and this same objection might also have been raised in connexion with the experiments of Karrer & Nägeli (1921), who obtained a maltose derivative in 80 per cent yield by the action of acetyl bromide on starch. Actually, the likelihood of this interpretation seemed small, since maltose itself under these conditions gave an identical product in comparable yield.

A rigid proof, however, of the preformation of maltose in starch was not forthcoming until Haworth & Percival (1931) subjected a fully methylated starch specimen in chloroform solution to the degradative action of this same reagent, acetyl bromide, under mild conditions (4 hours at 15°). From the mixed cleavage products a

disaccharide fraction was isolated which was definitely identified as maltose.[1] Its yield corresponded to 22·4 per cent of the original starch. Under identical conditions 2 : 3 : 6-trimethyl glucopyranose yields no traces of maltose derivatives.

Thus, it was established beyond doubt that one-fifth at least of the glucose units in starch pre-exist as contiguous pairs of glucopyranose units linked as in maltose.[2]

Subsequent investigations have served to establish the extensive and probably exclusive occurrence of the maltose linkage throughout the chain structure, and there is now little doubt that the conception of the starch molecule as a chain of recurrent α-glucopyranose units linked in positions 1 and 4 is substantially correct.

In considering the evidence in favour of this view, considerable weight must be attached to the fact that, in the extensive examinations of starch degradation products which have been made, no single example has so far come to light of a saccharide which has been shown to contain a β-linkage. Such purely negative evidence, however, cannot be considered entirely convincing.

We will consider therefore the two lines of investigation which, in combination, appear to render untenable an alternative view.

The first line of evidence has been obtained from the study of the progress of disintegration of starch (and cellulose) under mild conditions of acetolysis. As early as 1921, Freudenberg had applied this kinetic method to the study of cellulose and from reasoning based on the expected yields of cellobiose, assuming a haphazard fission of linkages, he was led to the view that cellulose is a long chain molecule consisting of equally linked glucose units.

The method has been extensively developed since then by Freudenberg (Reviews, 1931, 1936) and Meyer, Hopf & Mark (e.g. 1929), who have studied the progress of degradation of starch and cellulose under these conditions, as followed both by the liberation of reducing groups (hypoiodite) and changes in optical rotation. In each case, the velocity of hydrolysis of the polysaccharide itself is greater than the velocity observed for the corresponding disaccharide (i.e. maltose and cellobiose). On the assumption that fragments of intermediate chain lengths are hydrolysed at intermediate velocities,[3] there have been developed formulae to express the progress of the

[1] It was isolated in the form of methyl octamethyl maltobionate.

[2] Similar evidence for the pre-existence of the maltose linkage in glycogen was obtained (Haworth & Percival, 1931).

[3] In the case of cellulose the validity of this assumption has been established by the determination of velocity constants for cellotriose and cellotetraose.

degradation, as followed by either the chemical or optical method. These expressions, which embodied the assumption that the units and the linkages in each of the respective polysaccharides were entirely homogeneous, gave good agreement with the observed results. It is unnecessary to consider this work in detail, but one simple and significant conclusion deserves particular emphasis. We refer to the fact that velocity constants (and the calculated values for E, the critical increment, between 18° and 30°) exclude the possibility of the occurrence of appreciable numbers of glucofuranose (active glucose) units in either starch or cellulose. Furanoside linkages in general (cf. inulin, sucrose) are hydrolysed at an enormously higher rate than pyranoside linkages (of the order of 1000-fold), and the velocity constants throughout the degradation of starch and cellulose are of the order of those of pyranosides. This evidence accordingly points to the exclusive presence of glucopyranose units.

The second line of evidence, also developed largely by Freudenberg, will be considered separately.

The optical rotations of members of the starch series

Of great importance, from the point of view of amylase action, is the question as to whether the glucosidic linkages in starch are exclusively of the α (1–4) or maltose type as postulated in the Haworth-Hirst model. The presence of alternating α- and β-linkages has been postulated to explain certain mutarotational phenomena observed during the enzymic degradation of starch.

The most direct evidence bearing on this question has resulted from the formulation by Freudenberg *et al.* (1932) of the relationship between the optical rotation of a disaccharide and the rotations of higher saccharides containing exactly similar chain units, joined together by glucosidic linkages identical with that present in the disaccharide. This relationship is developed as follows.

The molecular rotation[1] of a polysaccharide, consisting of a chain structure built up of similar chain units, represents the sum of the rotational contributions of the individual chain members, the average value of which in a very long chain will be designated $[M]_\infty/\infty$. The molecular rotational values of the two dissimilar end units will naturally differ from this average value; these may be designated $[M]_a$ for the aldehydic end unit, and $[M]_e$ for the non-aldehydic end unit. On the other hand, it is argued, it would follow from W. Kuhn's

[1] The molecular rotation, $[M]$, is equal to the specific rotation, $[\alpha]$, $\times$ (molecular weight/100).

"principle of proximity and distance" that the rotational values for intermediate units would not differ appreciably from $[M]_\infty/\infty$. Thus, even in the case of the middle unit of a trisaccharide, which is directly contiguous with the two dissimilar end units, the altered positions (the aldehydic group, C_1, of one end unit and the hydroxylated C_4 of the other) are sufficiently distant to have no significant effect upon the rotation of the middle unit. Accordingly, on this additive basis, the molecular rotation, $[M]_2$, for the disaccharide amounts to $[M]_a+[M]_e$; the rotation $[M]_3$, for the analogous trisaccharide, $[M]_a+[M]_\infty/\infty+[M]_e$, and the rotations of higher saccharides will be simply related to these lower members of the series.

Thus, for a saccharide of n units, the molecular rotation

$$[M]_n=[M]_a+(n-2)\ [M]_\infty/\infty+[M]_e$$
$$=[M]_2+(n-2)\ [M]_\infty/\infty.$$

This relationship, which is referred to as Freudenberg's rule of optical superposition, forms a valuable means of constitutional enquiry in the polysaccharide group. Its basis is to some extent empirical, but there seems no doubt as to its validity for the approximate purposes to which it has been put.

It is to be noted that, according to the Freudenberg equation, a linear relationship is predicted between the values of $[M]_n/n$ and $(n-1)/n$ which characterize different members of homologous saccharide series.[1] This affords a convenient graphical method for testing the applicability of the formulation.

In the starch series there are available the rotatory powers (specific rotation in chloroform solution) of the fully methylated derivatives of starch, maltose and the range of starch dextrins described earlier. For each of these products chain length determinations by end-group assay have been reported (cf. p. 115). This permits the computation of the values of $(n-1)/n$ and $[M]_n/n$, the latter involving the calculation of $[M]_n$ from the molecular weight of the methylated product and the specific rotation.[2]

[1] This is evident when the Freudenberg equation is re-written in the form of the straight line equation, $y=mx+b$, viz.,

$$[M]_n/n=2\ ([M]_\infty/\infty-[M]_2/2)\ (n-1/n)+[M]_2-[M]_\infty/\infty.$$

[2] The rotatory powers of some of the starch products have been reported for the sodium D line, others for the mercury yellow (578) line. Values of $[\alpha]_{578}$ have been multiplied by 0·963 to obtain an approximate value for $[\alpha]_D$. The following are the values obtained:

n (chain length)	2	3	4	8	12	16–17	25–29
$[M]_D$ (in degrees)	394	823	1220	2980	4960	7300	11,000–11,500

These values have been plotted in Fig. 5 and in addition corresponding values (from Freudenberg (1933)) for members of the cellulose series.

The general linear distribution of the experimental points for both saccharide series establishes the generic relationship between starch

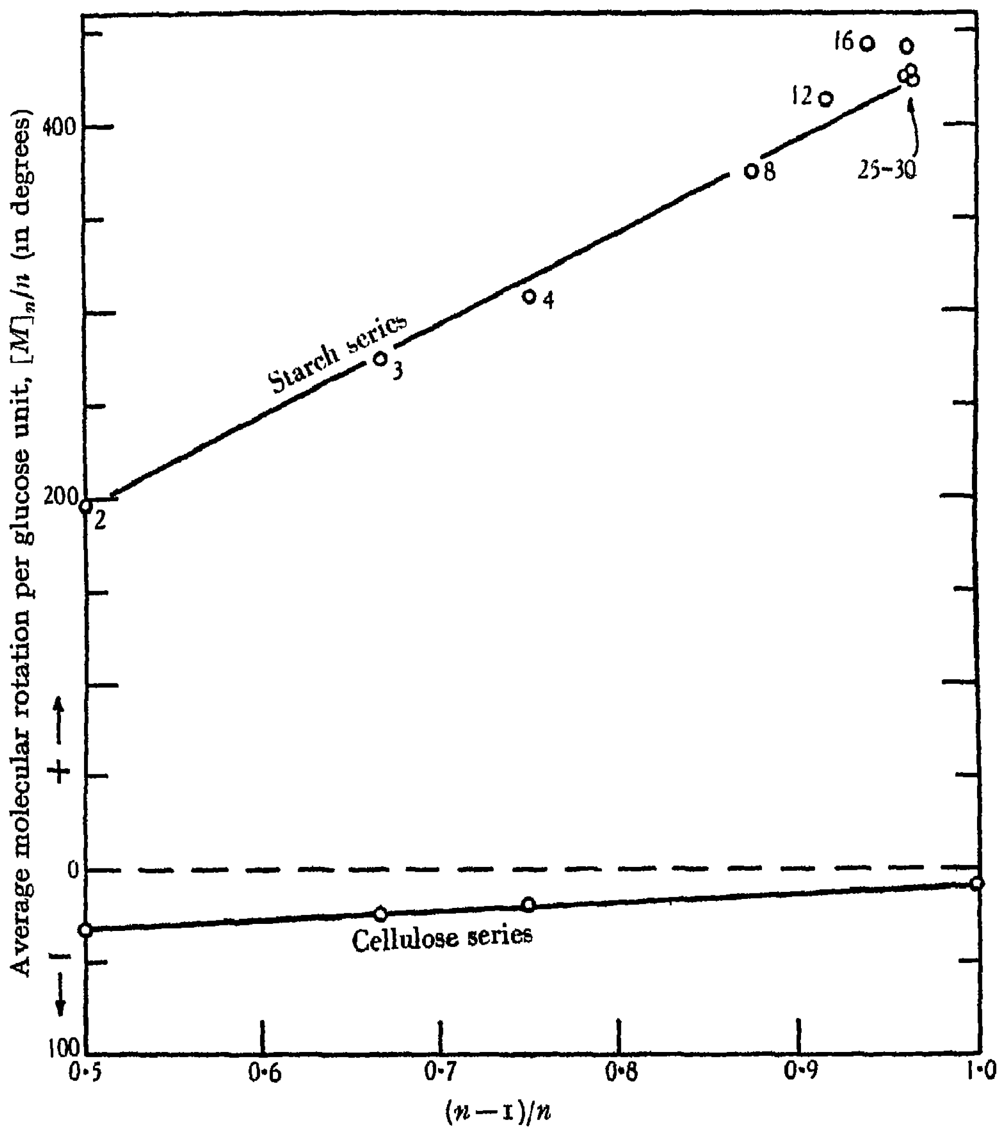


FIG. 5. Molecular rotations of fully methylated derivatives of starch and cellulose products (in chloroform solution). Chain length values for the different starch products are indicated for each point.

and maltose on the one hand, and cellulose and cellobiose on the other. The rotational powers of starch dextrins containing 3, 4, 8 and 12 units agree closely with the predicted values and are seen to conform with the rotation of the two extreme members of the series, maltose and starch. Moreover, the difference in rotational powers exhibited by the α-linked starch, and the β-linked cellulose series

emphasizes the stereo-isomeric relationship between these polysaccharides.

Some slight deviation from the linear relationship is apparent in the case of certain starch products of greater chain length. (It is to be noted that the rotations in these cases are higher than the predicted values; the presence of β-linkages would be expected to give lower values.) There is some evidence that this aberration may be the result of the formation of molecular aggregates, a tendency which is exhibited to a marked degree by α-amylodextrin ($n=16$).[1]

From this evidence it can be concluded with considerable assurance that the glucopyranose units, which constitute the chain molecule of starch, are present in the α-form, linked together as in maltose.

Aggregation of starch—amylose and amylopectin

The evidence leading to the conception of a chemical unit of starch with a molecular weight of about 5000 has been outlined. The fact must now be considered that determinations of particle size by a variety of physical methods indicate much higher values. Molecular weight values ranging from 20,000 to 200,000 have been reported, based on observations of viscosity (Staudinger & Eilers, 1936), sedimentation rate (Lamm, 1934), osmotic pressure (Samec, 1927; Carter & Record, 1936) and dialysis coefficient (Brinzinger & Brinzinger, 1931).

There is little doubt that the apparent discrepancy arises from the fact that starch in solution does not ordinarily exist in the form of its simple macromolecules, but that these are associated in varying numbers to form aggregates or micelles.

The postulation of a micellar structure for starch is by no means a new conception. It reflects the well-known fact that starch has a tendency to form colloidal aggregates of large dimensions. Some such assumption seems necessary to account for the phenomenon of "retrogradation" which characterizes starch and certain starch products. This term is used to denote the changes which occur when such products are stored in aqueous solution. The solutions become progressively more turbid and ultimately, under certain conditions, the process leads to the formation of microscopic particles, which,

[1] The specific rotation of α-amylodextrin in the free condition exhibits considerable variation, the values being related apparently to the physical properties of the different specimens. This will be discussed later. [Note added 11 March. The optical rotations of the methylated α-amylodextrin specimens which have been more recently examined (cf. p. 116) are not available for present consideration.]

from their concentrically banded appearance, have been called "artificial starch grains". Certain stages of the aggregation process are observable under the ultra-microscope (Samec, 1928).

While the intimate nature of this phenomenon remains obscure, the results of recent investigations have an important bearing on the problem.

It is possible, by various means, to separate starch into two fractions of widely different physical properties. These two fractions, commonly designated β-amylose and α-amylose, after Meyer (1895) or amylose and amylopectin, after Maquenne & Roux (1903), represent respectively, a fraction which is easily soluble in water and less viscous in solution, and a fraction which dissolves with difficulty, giving relatively opaque and viscous suspensions. The ability of native starch to form pastes with hot water was attributed by Maquenne and Roux to the presence of amylopectin.[1]

TABLE II. *Fractionation of potato starch by physical methods*

	Soluble fraction (amylose) %	Gelatinous fraction (amylopectin) %
Method of Gatin-Gruszewska (1908):		
Starch is swollen in 1·5 *N* soda. On neutralizing, amylopectin is precipitated	55–60	40–45
Method of Sherman & Baker (1916):		
Starch paste prepared at 80° in presence of NaCl, is separated by centrifuge	30–40	60–70
Method of Ling & Nanji (1923):		
Starch paste is frozen; after thawing the amylose is redissolved at 50°		
As used by Pringsheim & Wolfsohn (1924)	13	—
As used by Hirst *et al* (1932)	17	—
Method of Samec & Mayer (1921):		
Autoclaved starch paste is electro-dialysed. Erythroamylose is precipitated, amyloamylose remains in solution	17	83

It is clear that the products, designated amylose and amylopectin by different authors, are by no means identical and do not represent consistently the same portions of the starch substance. Thus the proportion of the two fractions varies enormously according to the method used for their separation, as will be seen from Table II.

[1] It is not proposed to enter in detail into the history of the terms "amylose" and "amylopectin" which have been used in different senses by different authors. Frequently amylopectin has been used to refer to the gelatinous hulls of envelope material present in starch paste although there has been no consistency in this regard. The reader is referred to the review by M. Schoen (1930).

It seems clear, therefore, that these two fractions, distinguishable on the basis of physical properties, must be to a large degree mutually interconvertible. There will be occasion to consider later the particular case of the amyloamylose fraction of Samec, which, in view of recent observations on its degradation by amylases, assumes a position of great theoretical interest.

We will now consider the hypothesis, which has been advanced by the Birmingham workers, regarding the relationship of the amylose and amylopectin fractions of the starch substance.

Hirst *et al.* (1932) examined specimens of amylose and amylopectin separated by the freezing-out method of Ling & Nanji (cf. Table II). They report a 17 per cent yield of amylose. Both the amylose and amylopectin so obtained retained the original phosphorus content of the starch (0·20 per cent calculated as P_2O_5).

As was mentioned earlier, end-group assay failed to reveal any difference between amylose and amylopectin (cf. p. 113). While no *chemical* difference was recognized between amylose and amylopectin, observations were made which offered a basis for interpreting the difference in their physical properties.

It was found in the first place that, just as amylose and amylopectin in the free condition are readily distinguishable by solubility in water and viscosity in solution, so too were the acetyl and methyl derivatives of these fractions distinguishable by their behaviour toward organic solvents (chloroform and acetone). Amylose, whether in the free state, or in the form of its derivatives, is more soluble and less viscous than amylopectin in the corresponding form.

Regardless of the method of preparation, acetylated amylose specimens dissolved easily in chloroform, giving mobile solutions, and it was not possible to separate them into fractions having different physical properties.

From amylopectin, on the other hand, by varying the conditions of acetylation,[1] a range of products was obtained, differing widely in physical properties. Thus, acetylated amylopectin specimens were prepared which, on the one extreme, were almost entirely insoluble in chloroform (exhibiting swelling without true solution) and, on the

[1] By the use of pyridine and acetic anhydride (4 days at 60–70° or shorter periods at 100°) highly viscous gelatinous acetylated amylopectin specimens were obtained. When the methods described earlier (p. 110), involving the use of SO_2 and Cl as catalysts, were used the products were more soluble and less viscous. Moreover, the viscosity of the product could be controlled by regulating the concentration of catalyst. With minimal amounts, viscous products similar to those obtained by the pyridine method were obtained, but on increasing the amount of catalyst, the products were more soluble and mobile.

other, specimens dissolving readily and forming mobile solutions. These latter products were indistinguishable from specimens of acetylated amylose.

On the removal of acetyl groups (by treatment with alcoholic soda), the regenerated amylopectin resembled in its physical properties the particular acetate from which it had been prepared. Thus, amylopectin acetates, which dissolved freely in chloroform, yielded regenerated products soluble in cold water and indistinguishable from amylose. Conversely, acetates of higher viscosity and smaller solubility gave rise to free amylopectin which was only partly soluble in water; in such cases the soluble portion had all the properties of amylose.

On methylating this range of acetates a continuous series of methylated amylopectin derivatives was obtained, varying widely in solubility and viscosity in solution. These methylated derivatives could be readily separated into portions having different physical properties.

The variation in physical properties of the amylopectin derivatives (and of free amylopectin regenerated from different acetates) was clearly not due to degradation in a chemical sense. The products showed negligible reducing power; the phosphorus content remained without appreciable change; and finally, viscous and mobile methylated specimens gave on hydrolysis the same yield of tetramethyl glucose, corresponding to a chain length of 24–30.

The conclusion was drawn, therefore, that amylose and amylopectin consist of identical macromolecules and that differences in solubility and viscosity depend upon the state of aggregation of these chemical units. Amylopectin is conceived to exist in an aggregated form, the interlocked macromolecules becoming hydrated in the presence of water with the formation of a micellar structure. In amylose solutions, on the other hand, the macromolecules exist in a less interlocked and more heavily hydrated state. The possible nature of such molecular associations will be discussed later (e.g. p. 129).

On this view, there should exist a continuous range of products (such as were, in fact, prepared) having properties intermediate between those of amylose and amylopectin. The retrogradation of free amylose in solution is interpreted as aggregation of the macromolecules through intermediate stages to the amylopectin condition. Conversely, the transformation of amylopectin into amylose, accomplished by de-acetylation of a soluble and mobile acetate, is

regarded as resulting from the disruption of the organized micellar structure.

It must be emphasized finally that these great alterations in physical properties were unaccompanied by appreciable changes in phosphorus content, which, in accordance with the views advocated by Samec (e.g. 1927) and others, has been considered to play an essential part in determining the viscosity of starch products (cf. p. 132).

Of considerable interest, from the point of view of molecular aggregation, are the observations of Baird *et al.* (1935) on a specimen of potato starch which had been subjected to a preliminary solubilization treatment. This modified starch, referred to as "simplified amylose", was prepared by treating starch grains with a hot dilute solution of hydrochloric acid in alcohol, the procedure being similar to that described by Small (1919) for the production of soluble starch with minimum degradation.

Soluble starch prepared in this way dissolves readily in hot water to form a relatively clear solution. When freshly precipitated from solution by alcohol, the product was found to be readily soluble in cold water. After standing for a few hours in an air-dry condition, however, it reverted to a form insoluble in cold water, from which it was inferred that re-aggregation had occurred.

The freshly precipitated, disaggregated product was acetylated in the presence of SO_2 and Cl, and then methylated. The chemical characteristics of this methyl derivative (which have already been given in Table I, p. 113) do not distinguish it from other methylated starches. On hydrolysis the yield of tetramethyl glucose indicated a chain length of 26 units.

In physical properties, however, the product is of considerable interest, since it showed exceptionally high solubility in chloroform and low viscosity in *m*-cresol solution. For comparison, a methyl derivative was prepared under identical conditions from unmodified starch, and it was found that the specific viscosity of this latter product was 4–5 times greater.[1]

It may be concluded that, as a result of the preliminary solubilizing treatment, the degree of molecular association of this starch specimen was considerably reduced without detectable alteration in chain length as revealed by end-group assay.

[1] The Staudinger limiting viscosity equation has been used in attempting a semi-quantitative interpretation of the viscosity of starch products in terms of chain length. There are grounds, however, for doubting the direct applicability of this formulation to the starch series (cf. Gallay, 1936) and this aspect of the subject will not be considered.

The molecular constitution of glycogen

It has long been held that starch and glycogen are closely related polysaccharides, and this view is substantiated by recent constitutional studies, which indicate that glycogen is built on a chain pattern identical with that of starch. The chemical molecule of glycogen, however, contains fewer glucose units.

The important fact has been established that there exist two distinct forms of glycogen, one containing 12 glucopyranose units in the chain molecule, the other 18 units.

The examination of glycogen by methylation and end-group assay was first carried out by Haworth & Percival (1932), using commercial samples of rabbit-liver glycogen. The results indicated a chain length of 12 units. This observation was confirmed and extended by Bell (1935), who prepared glycogen from rabbit and fish livers and found in each case a chain length of 12.

Later, specimens of 18-unit glycogen were encountered. This form, first reported in a preliminary announcement by Haworth (1935), was described in detail by Bell (1936), who discovered the interesting fact that the glycogen deposited in the livers of rabbits after the ingestion of galactose is of the 18-unit variety. The glycogen itself is composed exclusively of glucose units.

Despite the fact that the two forms of glycogen reveal this clear-cut difference in molecular structure, they are remarkably similar in general properties. The 18-unit glycogen is reported to be slightly more opalescent in solution than the 12-unit form and the optical rotation would appear to be slightly (but possibly not significantly) higher, $[\alpha]_D + 200°$ as compared with $+196°$. Otherwise they are not so far distinguishable except by the method of methylation and end-group assay. In each case the product exists in a highly aggregated colloidal state and exhibits extremely low reducing power to copper and hypoiodite reagents.

The problem of the aldehydic end unit of starch

While far-reaching conclusions have been based on the characterization of the non-aldehydic end unit of the starch chain and the frequency of its occurrence, the nature of the terminal unit at the opposite end of the chain remains an unsettled question. In view of the extremely low reducing power of starch, certain authors regard it as improbable that this terminal unit contains a free aldehydic group.

Starch, in the relatively slightly modified form of starch paste, exerts a reducing power which does not exceed (and is usually considerably less than) 0·5 per cent of the reducing power of maltose (0·5 per cent $R_{maltose}$), as determined by either alkaline copper or hypoiodite reagents. On a stoichiometric basis an open chain containing 30 units and terminating in a reducing unit would be expected to show 7 per cent $R_{maltose}$.

The case of glycogen affords a parallel and even more striking example of α-glucopyranoside chain structures which fail to exhibit the reducing properties which might be expected. The two known forms of glycogen consist of macromolecules containing respectively 12 and 18 glucose units; on a stoichiometric basis these should exhibit 17·2 per cent and 11·7 per cent $R_{maltose}$ respectively, whereas actually both glycogen forms are practically non-reducing (reducing powers of the order of 0·1–0·5 per cent $R_{maltose}$ by alkaline ferricyanide and 1–2 per cent by hypoiodite) (Bell, 1935, 1936).

Accordingly, it is clear that starch and glycogen, while they are not entirely non-reducing in character, exhibit only a small fraction of the reducing power which would be expected on the basis of their molecular dimensions, and it is necessary to assume, either that the terminal unit does not contain the free aldehydic group as in maltose, or that the reducing action of this grouping, if it is present, is in some way masked.

Conclusive evidence concerning this important aspect of the molecular structure of starch and glycogen is not so far available, and suggestions which may be put forward are to some extent speculative.

If it be assumed that the grouping about C_1 of this terminal unit exists in some modified form, it seems necessary to postulate a configuration which readily reverts to the glucopyranose form, since both starch and glycogen give practically theoretical yields of glucose on hydrolysis. This would seem to exclude, for example, the possibility of a carboxyl group in this position. Freudenberg however has suggested an anhydro-glucopyranose structure for this end unit, C_1 being linked with C_6 by an internal oxygen bridge with the removal of water; such a configuration would account for the practical absence of reducing power and would at the same time revert to glucopyranose on hydrolysis. This suggestion arose by analogy with the crystalline Schardinger dextrins (produced by the action of *B. macerans* on starch), which appear to consist of short α-glucopyranose chains terminated by this non-reducing end unit (Freudenberg, 1934).

As an alternative to the conception of an altered configuration of the terminal glucopyranose unit, the possibility has been mentioned that the reducing power may be masked. In this connexion, it is of interest that cellodextrins (i.e. β-linked glucopyranose chains) of molecular dimensions corresponding to starch and glycogen exert their full stoichiometric reducing action toward alkaline hypoiodite. There is at present no reason to postulate different behaviour in this respect for an α-linked molecular chain; and the invocation of some form of "steric hindrance" seems inadequate.

It should be recalled that both starch and glycogen exist in a highly aggregated condition. It is possible that the association of the chemical units may involve the terminal aldehydic unit in some form of combination which destroys its reducing properties.[1] If such were the case, it would be expected that the completely disaggregated product would exert its full reducing action. Some evidence in favour of this conception, derived from observations on the enzymic degradation of starch, will be discussed below.

PART II. GENERAL FEATURES OF STARCH DEGRADATION BY AMYLASES

STARCH AS A SUBSTRATE FOR AMYLASE ACTION

The action of amylase preparations on native starch is extremely slow and it is doubtful whether intact grains of at least certain starch varieties (e.g. potato) are acted upon by extractable amylases. This fact is of interest from both chemical and physiological view-points, since it raises the questions of the nature of the outer layer of the grain and of the way in which the degradation of starch grains is initiated in the living cell.

Microscopic observations during starch dissolution in plant tissues indicate that, in many cases, corrosion of the grains begins at localized surface areas. In germinating barley, for example, during the late stages of endosperm depletion, numerous empty hulls, representing the outer coats of the former starch grains, may be seen. These thin sac-like structures, as was reported by Brown & Heron (1879), are usually perforated by small holes, which appear to mark the points at which the initial corrosion occurred and through which the removal of the degraded content material was effected.

[1] In the case of the polysaccharide xylan, for example, it has been suggested by Haworth, Hirst and Oliver (1934) that the macromolecules may be united by chemical bonds linking the reducing end with a hydroxyl group of an adjoining chain.

Such observations leave no doubt that, in certain cases at least, starch grains are surrounded by a thin membrane which is degraded with difficulty by the starch-splitting mechanism as it operates within living tissues.[1]

Other lines of evidence indicate that the external membrane is different in its physical properties from the main bulk of the starch substance. Thus, intact starch grains are insoluble in cold water; they swell slightly but preserve their discreet form, even after prolonged soaking. Since both the so-called amylose and amylopectin portions of starch are hydrophilic in nature, the external material would seem distinct from these two fractions. By rubbing air-dry starch in a mortar, however, or by subjecting it to abrasion in a ball-mill, a product is obtained which swells instantly and, to a considerable extent, dissolves in cold water.[2] When a dry mount of such "cold water soluble" starch (potato) is flooded with water, it is possible to observe the rapid swelling and deformation of grains which were scratched and battered, but apparently unbroken, by the preliminary physical treatment. The considerable alteration in properties of the grains would appear, therefore, to be due to the rupture or cracking of the surface, the continuity of which in the normal grain prevents the full hydration and swelling of the interior material.

While the existence of an external membrane about the starch grain has been widely recognized, there seems as yet no convincing evidence as to whether or not its substance is chemically different from the bulk of the starch substance. Sande-Bakhuyzen (1925) considers it to represent a less hydrated form of the true starch substance; older workers have regarded it as a distinct polysaccharide and have designated it as starch cellulose (Brown & Heron, 1879). It represents a small proportion, probably less than 5 per cent, of the total dry substance (cf. review by Schoen (1930)).

[1] Horning & Petrie (1927), from a cytological study of different cereal grains during germination, came to the interesting conclusion that the corrosion of starch grains is associated with mitochondrial activity. They report that starch hydrolysis was invariably preceded, not only by an increase in the numbers of mitochondria, but by a migration of mitochondria to positions in contact with the starch grains.

[2] "Cold water soluble" starch, prepared by abrasion of starch grains, is readily separated in "amylose" and "amylopectin" fractions after suspension in water and centrifuging. (It is preferable to add a little alcohol to the dry material and then gradually add water in order to prevent the formation of lumps.) Potato starch under these conditions yields about 50 per cent of soluble "amylose", which remains in the supernatant liquid and the gelatinous "amylopectin" is thrown down (unpublished observations of the author). Alsberg & Perry (1924) reported a 60 per cent yield of soluble material from a similar preparation of wheat starch.

Starch paste

The oldest form of substrate is the starch "paste" prepared by heating an aqueous suspension of starch grains until gelatinization has occurred. The grains swell relatively suddenly during the heating to 30–50 times their original volume and in this swollen form they appear under the microscope as thin sacs (the distended external membrane) containing a fluid (the hydrated content material). Except with prolonged boiling, or mechanical breaking, many of these swollen grains persist unruptured in starch paste.

It was shown by Josza & Gore (1930) that by high speed stirring in the hot condition, followed by a similar treatment after cooling, much of the heterogeneity of starch paste is removed, and such "homogenized" preparations are recommended as a suitable substrate for observing the viscosity changes which result from amylase action.

Starch paste is convertible into a relatively mobile and almost water-clear solution by a short period of autoclaving (e.g. 30 min. at 120° C.), and preparations of this type have been used by Samec as a preliminary stage in his fractionations of starch by electrophoresis (cf. p. 123).

Soluble starch

Solutions of so-called soluble starch are now the most widely used substrates for amylase action. Soluble starch can be prepared from native starch grains by a number of methods. The Lintner process (consisting ordinarily in a 7-day treatment with 7·5 per cent HCl at room temperature) and the Small (1919) method (which involves a treatment with hot dilute alcoholic HCl) are perhaps the most generally used, and at the same time, the most desirable procedures. (In a number of other available methods the danger of induction of far-reaching degradation appears greater.)

By these treatments starch is converted into a form which dissolves readily in hot water. When the conditions of solubilization are properly controlled the resultant solutions are remarkably clear and exhibit only slight opalescence; such solutions are much less viscous than starch paste and on microscopic examination are seen to be free from the gelatinous bodies present in paste.

The intimate nature of the changes which occur during this remarkable transformation of starch are but little understood. The grain form remains without obvious change during the solubilization process, except that the surfaces lose the gloss characteristic of native starch. Moreover, the solubilized grains remain resistant to the action

of cold water and do not swell and disintegrate, as is the case with mechanically damaged grains. It is clear that effective solubilization of starch can be accomplished without appreciable hydrolytic degradation, since excellent specimens of soluble potato and other starches may be prepared which have very low reducing power (under appropriate conditions products obtained by the Small process exhibit 0·5–1 per cent $R_{maltose}$). It will be recalled, moreover, that Baird *et al.* (cf. p. 126) in preparing their "simplified amylose" used the Small solubilization process, and that this product, on methylation, gave an unaltered value by end-group assay.

The conversion of native starch into the so-called soluble form appears to consist essentially in the alteration of those obscure factors, which govern the association of the macromolecules into micelles.

CRITERIA OF STARCH DEGRADATION BY AMYLASES

The action of most amylases on starch results in (1) an initial rapid decrease in viscosity, particularly evident with starch paste; (2) the conversion of the substrate into products exhibiting altered iodine coloration (this normally changes from blue, through violet, rose and orange to the so-called achroic stage); (3) the appearance of reducing products. The changes in these three properties have formed, respectively, the criteria of the so-called liquefying, dextrinogenic and saccharogenic activities of amylases.

Viscosity change

An analogy has been drawn frequently between the enzymic liquefaction of starch and its conversion into soluble starch by chemical means.

Certain authors, arguing from the fact that some preparations are particularly potent in liquefying activity, and that the fall in viscosity may occur before the induction of appreciable changes in iodine coloration and reducing power, have regarded liquefaction as a process quite separate from the subsequent stages of degradation and one which is induced by a distinct liquefying amylase (cf. Chrzaszcz, 1911).

According to one view, the formation of the micellar structure of starch is conditioned by the presence of small amounts of inorganic constituents, phosphate in particular (cf. Samec, 1927; Malfitano & Catoire, 1925). It has been suggested that starch liquefaction by

amylases may consist essentially in the removal of such substances, with a resultant disruption of intermolecular associations.

Most investigators in the past have regarded as inconclusive, evidence of such general character. The recently reported isolation from barley of a specialized liquefying amylase is accordingly of great interest (cf. p. 139).

Iodine coloration

The intimate nature of the iodine colouring property of starch and starch dextrins remains obscure. The idea that the colour is due to the formation of definite chemical combinations with iodine has given place largely to the view that the uptake of iodine by starch products is a question of pure adsorption, dependent upon the colloidal state. Since Langmuir's work on gaseous adsorption, the essential difference between these views has become less readily definable and it is clear that the iodine colouring property must depend ultimately upon certain affinity residues in the molecular structure. This was pointed out by Bergman (1924) when he reported the fact that certain simple and crystallizable substances (the methyl cyclo-acetals of acetol and acetoin[1]) give blue-black compounds with iodine. This forms one of the few examples of substances of apparently crystalloidal character which form bluish compounds with iodine (e.g. certain derivatives of α- and γ-pyrone (Barger & Starling, 1915)). In a few other cases, an iodine coloration is given by substances (other than starch products) in which the property appears clearly related to their colloidal or semi-colloidal state (e.g. lathanium subacetate (Biltz, 1904), saponarin and cholalic acid (Barger & Field, 1912).

While cellulose under the action of sulphuric acid or zinc chloride develops a feeble blue coloration with iodine, the characteristic iodine reaction exhibited by starch products (and the closely related glycogens) has amongst polysaccharides a considerable degree of specificity. At present it seems not explicable solely on the basis of the physical state of these substances.

As an empirical index of starch degradation, however, the iodine

[1] These compounds contain an internal oxygen bridge of the type

$$—C(OCH_3)—C—.$$
$$\lfloor\_\_\_O\_\_\_\rfloor$$

This has given rise to the view (Samec, 1935) that the iodine colouring property of starch products depends upon the spatial arrangement of particular atomic groups (OH and C—O—C) in the chain structure.

colouring property has a value which is independent of conceptions of the mechanism of its production.

Whenever starch is degraded, whether by thermal decomposition (for example, in glycerol), by hydrolysis (acid or enzymic) or by the action of oxidizing agents (such as Fenton's reagent), a fairly typical and progressive transformation of the iodine coloration is induced. It is possible, moreover, to isolate dextrins, recognizable as fragments of varying complexity, which develop blue-violet, violet, red-brown and pale red colours with iodine.

The exact manner in which the iodine colour varies with the length of α-glucopyranose chain structures is not deducible from present information, and it is unlikely that chain length will be found to be the sole factor governing the particular hue of the iodine coloration.[1] There is, on the other hand, evidence which suggests that a certain minimal chain length is essential for the formation of the colour complex.

Fragments of the starch chain structure containing 6 units, or less, are devoid of iodine coloration. This follows from observations on the maltodextrins, and on the 6-unit dextrin of Waldschmidt-Leitz *et al.* 1932 (to be discussed later). Dextrins containing 8 and 12 units, respectively, have been reported to give a red coloration (cf. p. 115, Haworth, Hirst & Plant, 1935).

It would appear, therefore, that the minimal chain length which exhibits this property is either 7 or 8 glucose units and accordingly, that the so-called achroic stage of degradation corresponds to a point at which there remain no dextrins containing more than 6–7 glucose units.

Reducing power

The progressive increase in reducing power is clearly due to the liberation of reducing (aldehydic) groups by the hydrolysis of glucosidic bonds.

Ideally, a reduction value should indicate the proportion of the total linkages hydrolysed at any given stage. This direct interpretation is complicated to some extent, however, by the question as to whether aldehydic groups terminating fragments of different chain length exert the same reducing action. The practically non-reducing character of starch itself suggests the presence of a modified or masked terminal grouping and it is possible that any fragment

[1] This is suggested by a consideration of the colours developed by α-amylodextrin and the 12-unit dextrin of Haworth, Hirst & Plant, which exemplify retrograding and non-retrograding dextrin types (cf. p. 115).

containing this original terminal unit is also non-reducing. It is probable, however, that this group, originally non-reducing, gains its full reactivity as a result of certain types at least of enzymic degradation. The resultant increase in reducing power would, in this case, be the same as that caused by the hydrolysis of an interglucosidic linkage.

In spite of these reservations, a fairly precise indication of the number of linkages hydrolysed may be deduced and it is probable that, for chain fragments containing up to at least 6 glucose units, the reducing power is approximately theoretical.[1]

Owing to the fact that maltose is frequently the predominating reducing product which is formed (at least during the advanced stages of the enzymic degradation of starch) it has become customary to express the reducing power of starch digests and products in terms of maltose. These values are often reported as percentages of the theoretical quantity of maltose which would be formed from the complete transformation of the substrate into maltose.[2]

When maltose is the sole reducing product (as appears to be the case during starch degradation by the saccharogenic amylase of malt), this convention is devoid of ambiguity. The reducing value so expressed (as a percentage of the theoretical maltose) provides a measure of the extent of the transformation of starch into maltose. With the majority of amylases, however, maltose is by no means the sole reducing product, particularly during the early degradation stages. The significance of the conventionally expressed reduction value may obviously be very different in this case. Thus, an observed reduction value equivalent to 36 per cent of the theoretical maltose does not necessarily imply that this proportion of the substrate has been converted actually into maltose; the conversion of the whole of the substrate into a 6-unit dextrin would be expected to give about the same value.

While a reduction value gives an indication of the *number* of linkages hydrolysed, it affords no clue as to their relative *positions* in the original chain structure, nor of the nature of the products to which the reducing groups may be attached.

[1] It seems important to distinguish between alkaline hypoiodite, on the one hand, and alkaline copper reagents, on the other, in determinations of reducing power since the former reagent when used with suitable precautions (cf. Kline & Acree, 1930) reacts stoichiometrically with aldose groups. This cannot be claimed for alkaline copper reagents.

[2] 1 g. starch would yield theoretically 1·055 g. maltose, according to the equation $(C_6H_{10}O_5)_n + n/2\ H_2O + n/2\ (C_{12}H_{22}O_{11})$.

PART III. THE AMYLASE SYSTEM OF BARLEY AND MALT

The "component-enzyme" theory of malt amylase

The most important achievement in the field of amylase studies has been the gradual substantiation of the view, first put forward over 50 years ago, that malt amylase consists of a mixture of separate enzymes which induce distinctive types of starch breakdown.

The purely historical aspects of this development need not be considered here, but its basic significance cannot be over-emphasized. Already, by the end of the last century, there existed good evidence for the existence of two distinct malt amylases, the saccharogenic and dextrinogenic components, respectively, which were distinguished by the fact that the former produced considerable maltose from starch, without destroying the iodine colouring property, whereas the latter destroyed this property with the liberation of relatively few reducing groups. The precise nature of the chemical events underlying these two types of degradation constitutes a central issue of the amylase-starch problem.

In the light of this conception it has become possible to interpret the alteration in the nature of the amylase system which occurs during the germination process. The dormant barley grain normally contains only the saccharogenic amylase in an active condition, the dextrinogenic component appearing only after the beginning of germination.[1] Ungerminated barley now forms the most convenient source of the saccharogenic amylase.

In addition to these components, there is now no doubt of the existence of a specialized liquefying amylase (cf. p. 139). This enzyme is present in the ungerminated grain, but whether or not it increases in activity during germination has not been determined.

The separation of the saccharogenic and dextrinogenic components

(1) *The Ohlsson methods of differential inactivation.*

The investigation of Ohlsson (1926) provided reliable methods for preparing each of these components relatively free from contamination by the other, and at the same time offered a general hypothesis

[1] A more detailed discussion of investigators in this field seems unjustified in the present account. This aspect of the malt-amylase problem is of great interest since it sheds considerable light on the factors which enter into the control of enzymic activity during the remarkable transformations in physiological state, which are undergone during the phases of maturation, dormancy, and germination of the barley grain. The recent literature is discussed by Hesse (1934) and Oppenheimer (1936).

regarding the essential difference in their action on starch. This contribution may be regarded as the starting point of modern investigators.

The saccharogenic amylase, while relatively stable under acid conditions, is rapidly inactivated at elevated temperatures; conversely, the dextrinogenic amylase is inactivated by exposure to mild acidity, but is unusually thermostable. These general properties of the two enzymes were known to earlier workers, but Ohlsson, from a detailed study of their relative stabilities under a range of conditions, was able for the first time to prescribe treatments which cause practically complete destruction of one enzyme with only slight inactivation of the other.[1]

The method of selective inactivation as a means for isolating enzyme components is far from ideal on theoretical grounds. The possibility exists, although no clear-cut examples have come to light, that a single enzyme might be substantially altered as a result of the relatively drastic treatments involved. Accordingly, by a variety of such treatments, there might be derived a variety of forms of a single enzyme, exhibiting distinctive properties. For reasons of this order, the existence of two distinct amylases in malt has until recently been regarded by certain investigators as unproven or even doubtful (e.g. Sabalitschka & Weidlich, 1929; Waldschmidt-Leitz, 1929).

More recently, however, such doubt has been dispelled by the convincing weight of a number of separate lines of evidence.

(2) *The differential diffusion technique of Wijsman.*

Van Klinkenberg (1931) drew attention to the important observations of Wijsman which, although carried out in 1889, had received little notice owing to obscure publication[2] (Wijsman, 1889, 1890).

[1] The Ohlsson methods are as follows:

(*a*) The saccharogenic component is prepared by treating malt extract at 0° C., the acidity being adjusted to *p*H 3·3 with hydrochloric acid. The preparation retains 70–80 per cent of the original capacity to induce sugar production from starch while the dextrinogenic activity is reduced to 1–2 per cent of the original value.

(*b*) The dextrinogenic amylase is prepared by inactivating the saccharogenic component by 10 min. heating at 70° C., the acidity being that of the original extract (*p*H 6–7). The saccharogenic activity falls to 5–7 per cent of the original value while the preparation retains 60 per cent of the original capacity to induce the destruction of the iodine coloration.

The degree of selective inactivation resulting from these treatments, and the purity of the preparations, is higher than might be supposed from the figures cited. It is now clear that neither of the two amylases in isolation causes changes exclusively in the criterion used by Ohlsson for its estimation.

[2] The writer has not had access to Wijsman's main publication (1889) but has derived the ensuing information from van Klinkenberg (1931) and from the smaller paper by Wijsman (1890). The observations of Wijsman have been confirmed in nearly every detail by van Klinkenberg (*loc. cit.*).

Wijsman, by a simple technique, provided unequivocal evidence for the existence of two distinct amylases in malt. This demonstration was based on the fact that the saccharogenic amylase diffuses relatively rapidly into a stiff gelatin gel, whereas the dextrinogenic amylase diffuses very slowly into this medium.

When a small concentration of soluble starch is incorporated into the gel, the zones of action (and hence of diffusion) of the two enzymes became distinguishable on the addition of dilute iodine solution. This is due to the fact that the dextrinogenic amylase entirely destroys the iodine coloration, whereas the saccharogenic component, incapable of completely destroying this property, degrades starch to the stage of a dextrin which gives a mauve coloration with iodine. The diffusion fields of the two enzymes thus appear as colourless and mauve areas, respectively, in the blue-black background (beyond the field of action of either enzyme).

When droplets of malt extract were placed on gelatine-starch plates and the preparations, after 3–4 days in the ice-chest, were stained with iodine, the following diffusion picture was observed. There was typically a colourless centrum (marking the limits of distribution of the dextrinogenic amylase), surrounded by a mauve-staining ring (marking the wider field of action of the more diffusible saccharogenic enzyme).

The critical experiment in support of this interpretation was then performed. Small blocks of gelatine were removed from the outer ring region and transplanted to fresh plates. (This was done on plates which had not been treated with iodine, the positions being determined on controls.) It was found that such preparations gave rise to mauve-staining diffusion zones which contained no colourless centra. This observation showed conclusively the existence of a single enzyme in the outer zone and demonstrated therefore that the isolation of this component had been effected purely on the basis of the differential diffusibility of the two amylases.[1]

[1] In the absence of this critical demonstration, the mere observation of zones exhibiting different colorations would have been inconclusive. A preparation containing a single amylase of dextrinogenic nature, for example, would be expected to produce a diffusion picture exhibiting some degree of zonation of the iodine colour since the degree of degradation of the starch at the point of "inoculation" would be greater (owing to longer duration of action) than at the outer fringes to which the enzyme had subsequently diffused. In this case the zonation of the iodine colour would be merely a reflexion of the decreasing duration of action of the enzyme from the centre onwards.

Giri (1934) in an investigation of pancreatic and *Aspergillus* amylases by this technique appears to have completely overlooked this possibility. He concludes, without having performed the critical experiment carried out by Wijsman with malt amylase, that these two amylases are mixtures.

Having developed this method, Wijsman was able to confirm his conclusion in a number of ways and to make use of the method to gain an insight into various aspects of the problem of the two malt amylases which in certain directions remains unsurpassed.[1]

(3) *Differential adsorption.*

Waldschmidt-Leitz, Reichel & Purr (1932) reported the adsorptive separation of the two components from malt extract by the use of alumina C_γ. At *p*H 3·8 the saccharogenic amylase is adsorbed, together with a small amount of the dextrinogenic amylase, leaving only dextrinogenic amylase in the solution.

Holmbergh (1933, 1935) demonstrated a selective adsorption of the dextrinogenic amylase from weak (40 per cent) alcoholic solutions of the mixed amylases, by a number of different adsorbents. Particularly effective were native starch grains (rice and potato). The percentage adsorption on starch is little affected by acidity (within the range *p*H 4·4–7·6) but rises with decreasing temperature. The adsorbate is washed with 50 per cent alcohol containing 1 per cent maltose, and elution occurs in water.

By this means, a considerable degree of purification of the dextrinogenic amylase was achieved, both in freeing the preparation from the saccharogenic amylase and in increasing the activity/dry weight ratio.

The separation of the liquefying amylase

The early postulation of a distinct liquefying amylase has been mentioned above (p. 132). The isolation of this component has been reported recently by Waldschmidt-Leitz & Mayer (1935), and it has been designated "amylophosphatase" by these authors.

[1] Wijsman's discoveries included the demonstration of a partial fractionation of the two enzymes by alcoholic precipitation, and, foreshadowing the Ohlsson methods, differential inactivation by weak acids and heating, respectively.

He demonstrated that each of the two enzymes is able to attack undegraded starch, thus dispelling a view that the saccharogenic amylase acts exclusively on the products of the dextrinogenic enzyme. He described for the first time the resistant dextrin left by the completed action of the saccharogenic amylase. This substance he named erythrogranulose.

He found that the dormant grain contains only the saccharogenic component and that this is confined to the mealy endosperm tissue, the dextrinogenic enzyme appearing only after the beginning of germination. The latter amylase, originating first in aleurone layer and later in the scutellar epithelium appears to diffuse into the endosperm, its advent synchronizing with the beginning of starch grain corrosion.

The enzyme was separated from aqueous barley extracts by adsorption methods. It was first adsorbed, together with the saccharogenic amylase, on alumina C_γ. After elution, the saccharogenic enzyme was removed by successive adsorptions on kaolin, under appropriate conditions, leaving in the solution the new malt component whose properties will be considered in the succeeding Part.

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ON THE RELATION BETWEEN RESPIRATION AND FERMENTATION IN YEAST AND THE HIGHER PLANTS

A REVIEW OF OUR KNOWLEDGE OF THE EFFECT OF IODOACETATE ON THE METABOLISM OF PLANTS

By JOHN STEWART TURNER
Botany School, Cambridge

(With 3 figures in the text)

CONTENTS

I. Introductory discussion

This review is in the main a discussion of our knowledge of the effect of sodium monoiodoacetate on metabolism, and the bearing of this knowledge on the theory of respiration. It is opened, however, with a section which outlines the major hypotheses concerning the mechanism of fermentation and its relation to respiration, as these are relevant to the whole problem.

It is well known that many higher plants can survive in the absence of oxygen, producing alcohol and carbon dioxide. In some of these plants the final products of fermentation arise in the proportions obtaining for yeast fermentation, which satisfy the equation:

$$C_6H_{12}O_6 \rightleftharpoons 2C_2H_5OH + 2CO_2.$$

When the tissues of such plants are oxygenated (which is their normal state), oxygen is absorbed, and the products of respiration appear as water and carbon dioxide only; alcohol is no longer liberated in the cells.

Some of the higher plants live for only a short time in nitrogen and it is generally believed that these are rapidly poisoned by the products of their own fermentation, or that they die from a lack of working energy. In a few cases it has recently been demonstrated that some cells may live for many hours in nitrogen unharmed while producing no carbon dioxide at all. Thus Steier & Stannard (1936) show that although yeast cells when washed completely free of the sugar solution in which they were grown can carry out *endogenous* respiration at a very low rate, they do not, when undamaged, give out any appreciable quantity of carbon dioxide in nitrogen. The same has been demonstrated for some aerobic bacteria and for Chlorella cells under certain conditions. It is possible of course that these cells are carrying out, in nitrogen, a fermentation of the lactic type, producing no carbon dioxide; this seems in fact probable, as they remain unharmed in nitrogen. In any case it is not certain that the very weak *endogenous* respiration is concerned with a carbohydrate substrate. There is as yet no satisfactory evidence to show that higher plants, with or without added sugar, do not carry out fermentation in nitrogen. In this review then we shall discuss the possible relationship between fermentation and respiration in those tissues in which both processes may be demonstrated.

If we are to deal with fermentation in detail it will be necessary to describe very briefly the modern views on its mechanism. Until recently the schema of Neuberg was generally accepted. It stated that glucose, the substrate of fermentation, was broken down initially to methyl glyoxal. By a Cannizzaro reaction between this and acetaldehyde, pyruvic acid and alcohol were formed. The pyruvic acid regenerated acetaldehyde by decarboxylation, carbon dioxide being eliminated at this stage.

Recently this schema has been greatly modified following the discovery of the phosphate esters of the carbohydrates. Several tentative schemas have been put forward, and that set out in Fig. 1, based on papers by Meyerhof (1933), Meyerhof & Kiessling (1935) and by Parnas *et al.* (1935), illustrates the modern position sufficiently for our purpose. In this schema, methyl glyoxal plays no part in the cycle. Its place is taken by aldotriosephosphoric acid produced by the breakdown of hexose diphosphate. This triose compound reacts with

aldehyde to produce phosphoglyceric acid and alcohol. Aldehyde is constantly regenerated by the decarboxylation of phosphopyruvic acid, which arises from the phosphoglyceric acid. The phosphate radicle is passed back to the cycle through the carrying action of adenosin, and it combines with fresh glucose to give the hexose diphosphate.

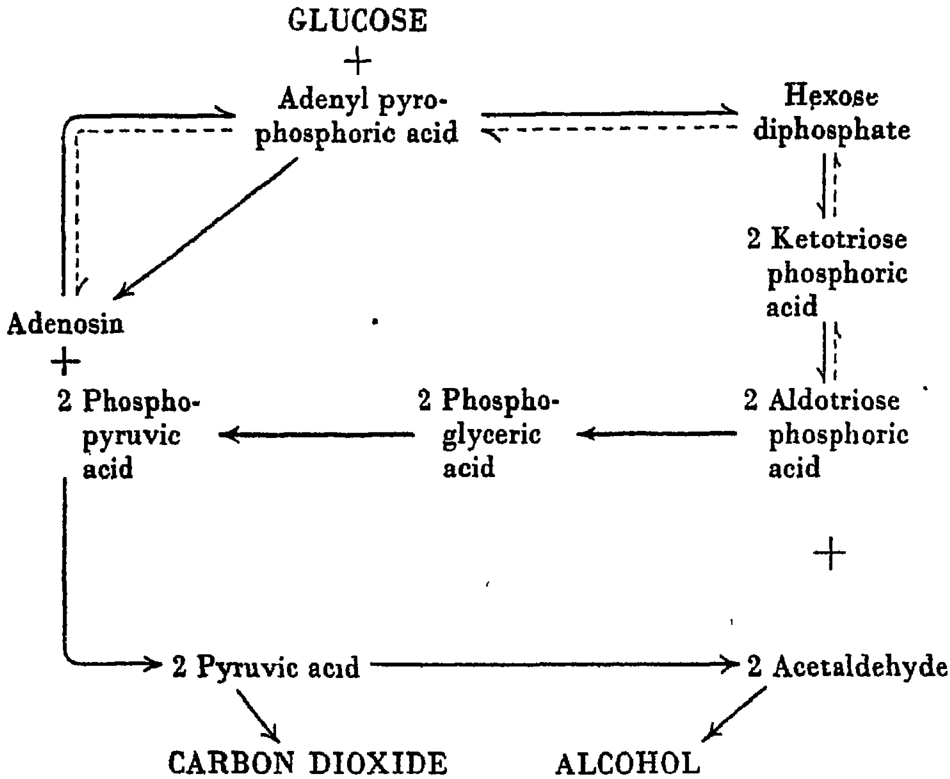

Fig. 1. New schema of fermentation, based on work in the schools of Meyerhof and Parnas.

In referring to the Neuberg schema and to that of Fig. 1 we have thought it convenient to use a new term. Much of the recent work on yeast fermentation is bound up closely with similar work on animal tissue, and this has led to some confusion in the use of the word *glycolysis*. Animal tissue and yeast under certain conditions can produce lactic acid from various substrates such as glycogen, hexoses and methyl glyoxal. This process is widely known as *glycolysis*; recently the word *glucolysis* has also been used to indicate specifically the production of lactic acid from glucose.

In the course of normal alcoholic fermentation by yeast and higher plants, lactic acid is not produced in appreciable quantities but the word glycolysis has often been loosely used to mean the production of a 3-carbon compound from a hexose or a disaccharide. In the present paper the word *triosis* has been substituted. It is defined thus:

Triosis—the production, by the splitting of a hexose, or some phosphorylated product of a hexose, of an unspecified 3-carbon compound which is not lactic acid.

Triosis is considered to be the first stage of fermentation in plants. On the Neuberg schema it implies the production of methyl glyoxal from glucose. In the schema of Fig. 1, it means the production from glucose, in the presence of adenylpyrophosphate, of aldotriose phosphoric acid. When the details of the chemistry of fermentation have been more fully elucidated, no doubt some more specific term will become applicable, but meanwhile, in view of the confusion resulting from the indiscriminate use of the word *glycolysis*, the coining of a new term is perhaps justifiable.

Wherever it is clear from the context that an author means by glycolysis the process we have defined as triosis, we have substituted this word for his in discussing his work.

During both fermentation and respiration plants lose carbohydrates, and it is generally assumed that carbohydrates are the ultimate substrates of both processes, except possibly in the case of certain sugar-starved tissues. A correlation, such as has often been demonstrated, between the rates of respiration and fermentation in any one organism under a set of different conditions does not constitute proof that the two processes have any part of their mechanisms in common. Nevertheless respiration and fermentation may be regarded as connected at least in the sense that when plant tissue is fully aerated, one of the products of fermentation—alcohol—no longer appears as end product. Fermentation is thus either suppressed by or replaced by respiration, a phenomenon often referred to as the Pasteur effect. Several explanations of this effect have been put forward, and we shall deal with them briefly under two headings.

(1) *The Pfeffer hypothesis, modified by Blackman*

The Pfeffer hypothesis, which has been widely accepted by plant physiologists, assumes that oxygen attacks one of the intermediate products of fermentation, water and carbon dioxide being the sole end products of the reactions. It was originally put forward to explain the Pasteur effect and the correlation observed between the rates of fermentation and respiration.

It application is simplest in those few cases known where the rates of sugar loss in nitrogen and in air are initially the same.

By this we mean that the initial rate of CO_2 production in nitrogen (*INR*) is one-third of the rate (*OR*) during an immediately preceding period of respiration. Assuming the equation of fermentation to be that on p. 142, this indicates that sugar is broken down at the same rate just previous to and just subsequent to a transition from aerobic to anaerobic conditions.

Thus it has been shown that the ratio of *INR* to *OR* is approximately 0·33 in germinating barley seeds (Barnell, 1937), and in germinating Fagopyron seeds (Leach, 1936). In these cases it seems reasonable to assume that $3 \times INR$ gives an estimate of the rate of triosis in the period of *OR*, that this rate is a factor limiting both respiration and fermentation, and that the Pfeffer schema holds, the whole of the products of fermentation being oxidized in respiration.

Now in many tissues the initial rate of sugar loss in nitrogen is significantly greater than in air. Among those plants investigated, this holds for apples (Blackman & Parija, 1928), yeast (Meyerhof, 1925), cherry laurel leaves, tomato fruits (Gustafson, 1930) and carrot tissue (Turner, 1937). At first sight it might be thought that the rate of oxidation in such tissues was the limiting factor, and that in spite of a high triosis rate (indicated by a high *INR*), only a low oxidation rate could be maintained on account, say, of enzyme deficiency. On this view, however, we should expect a piling up of triosis intermediates or of fermentation products in air, such as cannot usually be demonstrated. In the case of yeast it is true that some aerobic fermentation takes place, but even so the total sugar loss in air is less than in nitrogen. On the Pfeffer schema only two explanations of this seem possible.

(*a*) *The "permeability effect".*

We can assume that the rate of triosis is very rapidly lowered by the presence of oxygen and raised in its absence. The *INR* rates (determined by the method of Blackman (1928)) would then be no measure of the triosis rate in the preceding period of respiration. In recent papers by Dixon & Holmes (1935) it is suggested that oxygen slows up the rate of triosis by preventing in some way the ready access of the substrate to the enzyme concerned. They would therefore explain the results of Blackman & Parija (1928) by assuming that as soon as the apples are transferred to nitrogen the rate of triosis rises to its maximum. In this way they account for the *Pasteur reaction*, by which they understand the lowering of the rate of sugar loss on a transference of a tissue from nitrogen to air. Presumably in the above quoted cases, in which no such Pasteur reaction may be demonstrated, the permeability effect is lacking.

If it be found necessary, in explaining the Pasteur reaction, to postulate a reduction in the rate of triosis in air, then a possible mechanism for this is suggested on examination of the schema of Fig. 1. Here the breakdown of the hexose is dependent on a supply

of phosphate by adenylpyrophosphate, which is constantly regenerated by the reaction between adenosin and phosphopyruvic acid. If we assume on the Pfeffer schema that oxygen attacks the aldotriosephosphoric acid in respiration, it may be that the phosphate thereby set free cannot be readily transferred to fresh glucose. Unless some system exists in the oxygenated cell as efficient in returning the phosphate set free as is the anaerobic adenosin system, then the rate of triosis should fall in oxygen. There would be no need then to suggest a permeability effect.

(*b*) *Oxidative anabolism.*

Blackman (1928) considered the possibility of a rapid effect of oxygen on triosis, and he discarded it. He did in fact suggest that oxygen possibly slowly increased the rate of triosis or of some reaction previous to it, but he satisfied himself that an effect of oxygen on triosis, rapid enough to explain the Pasteur reaction, was improbable

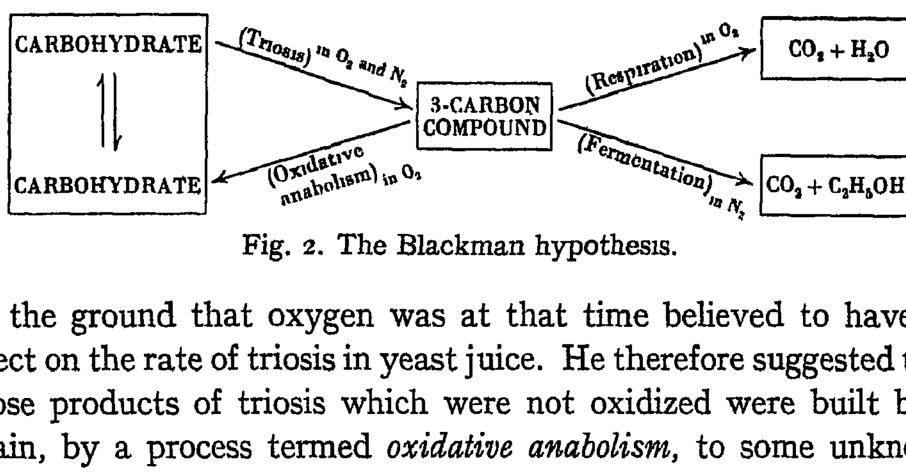

Fig. 2. The Blackman hypothesis.

on the ground that oxygen was at that time believed to have no effect on the rate of triosis in yeast juice. He therefore suggested that those products of triosis which were not oxidized were built back again, by a process termed *oxidative anabolism*, to some unknown substance, probably a carbohydrate. His hypothesis is therefore a modification of that of Pfeffer; it is summarized in Fig. 2, and will be referred to as the Blackman hypothesis.

(2) *The Lundsgaard hypothesis*

Quite a different view on the whole subject is taken by many workers in animal physiology and biochemistry; this view has long been held by Boysen Jensen (1931) and it has recently been brought into prominence by Lundsgaard (1930). We shall term it "the Lundsgaard hypothesis". It states that sugar breakdown in fermentation bears no relation to its breakdown in respiration. The desmolytic processes known to occur in yeast fermentation are thought to play no part in preparing the complex sugar molecule for oxidation.

Such an hypothesis would account very simply for the existence of *endogenous respiration* noted above. It will prove difficult on this

view, however, to explain those cases where the rates of sugar breakdown in air and nitrogen are initially the same. In these cases, as well as in those in which the sugar loss in nitrogen is initially greater than in air, the hypothesis must also offer some explanation of the cessation of fermentation in oxygen. This has been done as follows.

(1) Workers have usually followed the lead of Meyerhof (1925), who postulated that with the aid of the energy obtained from respiration the products of fermentation, final or intermediate, were built back into carbohydrate. This process, usually referred to as the Meyerhof reaction, is precisely the same as the oxidative anabolism postulated at about the same time by Blackman. On the Lundsgaard hypothesis however two alternative explanations of the Pasteur effect are possible:

(2) In suggesting the existence of a "permeability effect", Dixon & Holmes (1935) tacitly adopted the Pfeffer hypothesis. We might

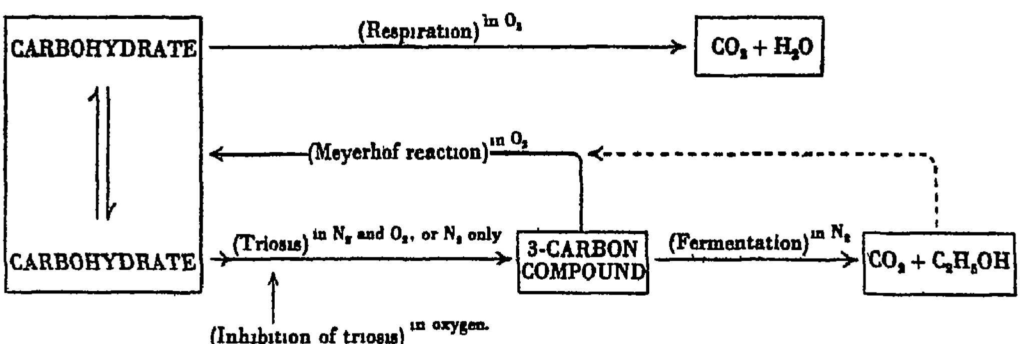

Fig. 3. The Lundsgaard hypothesis.

apply their theory more fully however and suggest that in oxygen the enzymes of triosis become not only partially but completely protected from their substrates.

(3) Lipmann (1933), who adopts the Lundsgaard hypothesis, explains the Pasteur effect by assuming that oxygen, working through a carrier, inhibits the enzyme of triosis. The inhibition is reversible. It is interesting to find this theory based on experiments which show that oxygen does retard triosis in yeast juice so long as a suitable carrier is present (see p. 147, line 16).

Either of the alternatives (2 and 3) carries with it the conclusion that an aerobic cell must contain a number of enzymes (those concerned with fermentation), which are never brought into action unless the cell is placed under anaerobic conditions.

The Lundsgaard schema, with its various alternative explanations of the Pasteur effect is represented schematically in Fig. 3.

We have now outlined the major difficulties to be met with in the application of either of the respiration hypotheses to the known facts. It is not the purpose of this review to discuss in detail the views of Lipmann and of Dixon & Holmes, or to offer evidence for or against the existence of oxidative anabolism. The essential difference between the hypotheses of Blackman and Lundsgaard may be put thus:

A. The products of triosis are partly or entirely used up in oxidation during respiration (Blackman).

B. The products of triosis are not used up in oxidation during respiration, which is a process entirely independent of fermentation (Lundsgaard).

This brings us to the work on which statement B is based. Lundsgaard (1930), in investigations on the effect of sodium monoiodoacetate on metabolism, has brought to light phenomena which he regards as demonstrating the validity of the hypothesis to which we have given his name. The present author, while not contesting the importance of Lundsgaard's observations, believes that the results so far obtained with iodoacetate may be adequately accounted for on the Blackman hypothesis. In his opinion, discrimination between the opposed hypotheses, A and B, has not been accomplished as yet by the use of iodoacetate.

II. Lundsgaard's preliminary experiments

In 1929 E. Lundsgaard discovered that sodium monoiodoacetate inhibited glycolysis in muscle, a discovery which later resulted in great modifications in the theory of muscle metabolism. In 1930 Lundsgaard went on to state that iodoacetate was equally of value in discriminating between the rival theories of plant respiration. He used iodoacetate in work on yeast, and drew the following important conclusions which are set out below in order that they may be dealt with in turn in our subsequent discussion.

(1) That sodium monoiodoacetate in certain concentrations completely inhibits the fermentation of yeast or zymase.

(2) That (1) is a direct consequence of the inhibition of "glycolysis" (triosis); this inhibition is assumed to occur because (*a*) iodoacetate certainly inhibits true glycolysis in muscle, and (*b*) in iodoacetate-poisoned yeast under anaerobic conditions, no loss of glucose occurs and no accumulation of intermediates may be detected. The same applies to zymase preparations.

(3) That certain concentrations of iodoacetate which inhibit F, when applied to aerated yeast have little or no effect on the rate of

respiration as measured by oxygen uptake. This differentiation in the effect of iodoacetate on F and R will be termed, for convenience, the "Lundsgaard effect", and the concentrations concerned are the "differentiating concentrations".

(4) That as a result of (2) and (3) we must abandon any hypothesis of type A which assumed a genetic connexion between F and R, for on such we should expect inhibition of triosis to be followed by inhibition of both F and R. Lundsgaard therefore favoured a hypothesis of type B, as set out on pp. 147, 149.

(5) That iodoacetate lowers the R.Q. of yeast from its normal high value of about 1·4 to nearly 1·00. This effect is interpreted as due to the inhibition of the fermentation which normally accompanies respiration in aerated yeast, and which is responsible for the normal high R.Q.

(6) That if we inhibit the fermentation of yeast by iodoacetate at the differentiating concentration, and then return the sample to aerobic conditions, the normal process of respiration is resumed, and that the existence of this effect further strengthens the evidence in favour of the Lundsgaard hypothesis.

As will appear in the course of this review, Lundsgaard has recently had occasion to modify some of these claims, but his interpretation of the effect of iodoacetate on respiration and fermentation remains essentially unaltered. We have thought it best to review his work from the beginning, as somewhat uncritical references to his early papers are now to be met with in works on plant respiration.

It is obvious that Lundsgaard's case rests in the first place on his assumption (2) that iodoacetate acts by inhibiting triosis. It must be stressed here that the Lundsgaard effect would be easily explicable on Blackman's hypothesis if it could be shown that iodoacetate had no direct effect on triosis, but acted on some later stage of fermentation. Lundsgaard had shown that iodoacetate inhibited glycolysis in muscle, and he assumed that it also inhibited glycolysis (triosis) in yeast. Boysen-Jensen (1931) also made this assumption, but it rests on no clear evidence. In fact, Nilson *et al.* (1931), who confirmed the Lundsgaard effect, soon pointed out the obvious fallacy in the argument. The inhibition of the later stages of a series of linked reactions might in itself be sufficient to cause apparent direct inhibition of the early stages also. Inhibition of carboxylation, for example, might result in the indirect but complete inhibition of phosphorylation, and there is no need to assume that this would be accompanied by the piling up of intermediate products in any measurable quantities.

The present author believes, however, after examination of more recent work, that Lundsgaard was right on this point. There is as yet no clear evidence against the view that iodoacetate does inhibit triosis directly. It would simplify matters to state this conclusion, and to pass on to consider other possible fallacies in Lundsgaard's work. Nevertheless, we shall first review in detail the work concerning inhibition of fermentation by iodoacetate, as it has important bearings on the general theory of the mechanism of fermentation.

III. The mechanism of fermentation inhibition by sodium monoiodoacetate

The work with iodoacetate, apart from its interest in connexion with Lundsgaard's hypothesis, is of importance in helping to unravel the complex problem of hexose breakdown. At the present time two opposed theories of triosis and fermentation are under discussion; each is associated with its own theory of fermentation inhibition by iodoacetate.

(*a*) *The Neuberg schema, and the coenzyme theory of inhibition*

In the well-known schema of fermentation first proposed by Neuberg, methylglyoxal plays an important part. It is supposed that this substance is produced on the initial triosis of glucose, and that by a Cannizzaro reaction with acetaldehyde it gives rise to pyruvic acid. Yeast contains an enzyme, methylglyoxalase, which catalyses *in vitro* the transformation of added methylglyoxal to lactic acid. Exactly what part this enzyme plays *in vivo* is obscure, but it seems to be generally assumed that it catalyses the above Cannizzaro reaction, for normal yeast does not produce lactic acid in fermentation.

Dudley (1931) was the first to discover that iodoacetate in strong concentrations inhibited glyoxalase activity *in vitro*. He suggested that this inhibition was the primary cause of the failure of poisoned muscle to produce lactic acid. As glyoxalase is also present in yeast (Platt & Schroeder, 1934) and the higher plants, it was natural to suppose that its inhibition was also responsible for the inhibition of fermentation.

The mechanism of the inhibition of glyoxalase by iodoacetate was soon discovered. Lohman (1932*a*) showed that reduced glutathione (GSH) was the coenzyme of glyoxalase, and numerous workers (Bersin, 1932; Dickens, 1933; Quastel, 1933; and Schroeder *et al.* 1933) have proved that the destruction of GSH by iodoacetate, which occurs *in vivo* and *in vitro*, is responsible for the lack of activity of

glyoxalase poisoned with iodoacetate. Thus Lohman (1932 *a*) showed that glyoxalase itself was unharmed by iodoacetate, and that its activity could be restored in a poisoned sample by the addition of sufficient reduced glutathione.

Dickens (1933 *a*) then showed that the velocities of reaction between GSH and chlor-, brom- and iodoacetates are as 15 : 9 : 0·15, which corresponds with the activities of these compounds as inhibitors of muscle glycolysis. He also concluded that the concentration of iodoacetate required to inhibit lactic acid production from methylglyoxal, *in vitro*, was of the same order as that required to inhibit tissue glycolysis.

Several recent papers supply evidence in support of the suggestion that GSH is of great importance in glycolysis and in yeast fermentation. Thus Quastel & Wheatley (1932) state that a high external concentration of GSH or of cysteine increases the rate of aerobic fermentation in yeast to the anaerobic level, with only a slight effect on the respiration rate. They believe that the SH compounds control the quantitative relations between fermentation, respiration and oxidative anabolism. Their observations, which remain so far unconfirmed, suggest that destruction of GSH by iodoacetate is of importance, whether or not iodoacetate inhibits triosis independently of GSH. The view of Waldschmidt Leitz (1930) that SH compounds have a regulating action on metabolism is also of interest in this respect.

Schroeder *et al.* (1933) have shown that the destruction of GSH in yeast by iodine runs more or less parallel with the suppression of fermentation, and this again suggests that glutathione, possibly in its role of coenzyme, is necessary to a system carrying out alcoholic fermentation. Nevertheless, these same workers have offered the most convincing evidence against the theory that iodoacetate inhibits fermentation merely by destroying the coenzyme of glyoxalase.

They showed that a concentration of iodoacetate of 1/5000 ($1{\cdot}075 \times M^{-3}$) inhibited yeast fermentation completely, but destroyed at the same time only 30 per cent of the total GSH content of the cells. At *p*H 4·5 a concentration of 1/50,000 ($1{\cdot}075 \times M^{-4}$) inhibited fermentation completely while destroying only 16 per cent of the GSH. Moreover no reactivation of the fermentation-enzyme system resulted on the addition of reduced glutathione; whether the glutathione entered the cells is not known. It was pointed out that the relatively slight alteration in the GSH concentration brought about by iodoacetate could not be responsible for the inhibition of fermenta-

tion, for it had already been demonstrated that partial destruction of GSH by *iodine* is not accompanied by full inhibition of the fermentation. Thus unless these experiments are faulty in some respect (i.e. in the method of estimation of the GSH), we are forced to conclude that although the GSH-iodoacetate reaction may introduce complications, it is not, in itself, the sole cause of the inhibition of fermentation by iodoacetate. This is the conclusion already reached on other lines by the Meyerhof school.

(b) The Meyerhof-Parnas schema and the enzyme theory of inhibition

We have already outlined in Fig. 1 the Meyerhof-Parnas view of fermentation. Part of the evidence in support of this was obtained in work with iodoacetate.

Thus Lohman (1933) showed that lactic acid production from muscle glycogen takes place in the complete absence of glutathione. Iodoacetate, however, inhibits this GSH-free glycolysis, and the addition of GSH to the poisoned muscle does not increase the rate of lactic acid production. Moreover, while muscle tissue will produce lactic acid from glycogen, from hexose phosphate and from methyl glyoxal, and all these reactions are inhibited by iodoacetate, the last of them is the least sensitive. Lohman therefore concluded that the action of iodoacetate with GSH was of secondary importance, and the iodoacetate must act in some other way in suppressing glycolysis. He believed that it inhibited directly some enzyme concerned in either the formation or the breakdown of the phosphoric esters.

Other workers are in agreement with this conclusion. The preliminary work of Yamasaki (1930) was inconclusive, but Haarman (1932), using animal tissue, has provided more complete and satisfactory evidence. He showed that lactic acid production from each of the substrates, methylglyoxal, pyruvic acid, hexose phosphates, glycerin phosphates and organic acids is less affected by iodoacetate than is the direct glycolysis of glycogen. He came to the "fairly safe conclusion" that the iodoacetate acts on some process previous to the formation of the 3-carbon compound.

More recently Meyerhof & Kiessling (1935) have published data which at first sight tells against the above. They have shown that iodoacetate does not inhibit the fermentation of phosphoglyceric acid to carbon dioxide and acetaldehyde, but that it does prevent the interaction of the aldotriose ester with acetaldehyde (see Fig. 1). Now this is a reaction subsequent to triosis proper, and if this can be

shown to be the only place of attack of iodoacetate it will itself explain the Lundsgaard effect (for we could assume on the Blackman hypothesis that oxygen attacks the triose esters in respiration). But Meyerhof & Kiessling themselves offer no evidence to prove that iodoacetate does not also inhibit triosis directly, and they state:

Das schliesst aber nicht aus, dass gewisse Teilreaktionen weder durch Fluorid noch Jodessigsäure gehemmt werden, und zwar gilt dies, ebenso wie für die carboxylatische Spaltung der Brenztraubensäure, auch fur einen Teil unserer Reaktion, nämlich für die eigentliche Umesterungsreaktion.

We conclude for the present then, following Lundsgaard, Lohman & Harmann, that iodoacetate may be considered to act directly as an inhibitor of triosis. We have abandoned the view that it does so by reaction with GSH, but we still have to explain the actual mechanism of the enzyme of triosis.

Lohman believes that iodoacetate acts on the enzyme "glycolase" ("Triosin") directly, not by destroying its coenzyme. A mechanism for such enzyme inhibition by substances of the type of iodoacetate has been suggested by Rapkine (1933). This is of importance as it tends to harmonize the two opposed explanations of the action of iodoacetate. Rapkine showed that iodoacetate reacted with the -SH groups present in plant proteins after reduction. This reaction has since been used by Mirsky & Anson (1934) as a means of estimating the -SH groups present in extracts of plant proteins. Rapkine suggested that the inhibition of triosis, at whatever stage it occurred, is due to the interaction of iodoacetate with the -SH groups which are thought to form an essential part of the enzyme or enzyme carrier concerned in triosis.

As an example of a somewhat similar mechanism of inhibition we may quote the results of Hellerman & Perkins (1934), who have shown that both urease and papain are reversibly inactivated by catalysed oxidation, and by certain metallic and mercurial-organic derivatives. Crystalline urease is known to contain -SH as an integral part of its molecule (Sumner & Poland, 1933), and it is suggested that the inhibition involves the formation of substances of the type En-SS-En, and En-S-Cu, regarded as inactive forms of the enzyme which may be re-converted to the enzyme En-S-H by suitable treatment.[1]

[1] We have not thought it worth while here to deal in detail with a coenzyme theory of inhibition put forward by Barrenscheen and his co-workers (1931), and supported by Zuckerkandl & Klebermass (1931), as a convincing explanation of their results, on other lines, has been given by Schroeder *et al.* (1933 *a*).

This side of the subject may eventually be clarified by work with derivatives of iodoacetate. Mowat & Stewart (1934) have stated that although iodoethyl alcohol slowly inhibits tissue glycolysis, it does not, like iodoacetate, react with the -SH group. Goddard & Schubert (1935), however, contradict this, and show that the iodoethyl alcohol does in fact react slowly with iodoacetate. Genevois (1933) and his school have examined the effects of a whole range of substances related to iodoacetate. The reactions of these substances with the SH radicles have yet to be explored.

It is still doubtful whether the schema of Parnas and Meyerhof applies to all tissues, and it would be premature to abandon the older theory of Neuberg. Ashford & Holmes (1929) think that phosphorylation plays no part in glycolysis in brain tissue and tumour, processes which are nevertheless inhibited by iodoacetate. Recently, too, Geiger (1935) has shown that the glycolysis of lactic acid from the glycogen of the brain is different from the *glucolysis* of lactic acid from glucose, and he holds that the Meyerhof cycle applies only to true glycolysis in muscle. He believes that methylglyoxal and glutathione play a part in animal *glucolysis*. Whether this applies to yeast triosis is not known.

In any event it is of importance to establish whether the inhibition of fermentation by iodoacetate is reversible or not. The combination of iodoacetate and glutathione *in vitro* to form a thio ether is generally regarded as irreversible. Lohman (1933) and also Ehrenfest (1933) state that the iodoacetate inhibition of fermentation in yeast cannot be reversed by the washing away of the iodoacetate, or by the addition of coenzymes. Sturm & Schulz (1933), however, in one experiment, obtained complete recovery of the fermentation in iodoacetate-poisoned yeast, by changing the *p*H from 6·0 to 7·3. Schroeder *et al.* (1933*a*) also record that occasionally slight reactivation of the fermentation took place after the addition of carbonate to the poisoned yeast. Cayrol & Genevois (1931) state that when yeast has been in contact with iodoacetate for "only a few hours", the normal rate of F is restored by washing the yeast in fresh buffer. They give no protocols in support of this statement. Finally Ehrenfest (1933) has stated that a period of alcohol oxidation by yeast, whose fermentation has been inhibited by iodoacetate, is followed by a restoration of the power of fermenting sugar. Confirmation of this important statement is lacking.

On the whole we conclude provisionally that the evidence favours the view put forward by Lundsgaard, that iodoacetate causes inhibi-

tion of fermentation by its direct combination or reaction with an enzyme system concerned in triosis, the first stage of fermentation. It is possible that the iodoacetate works by reacting with the fixed -SH groups of proteins, and the possibility is not excluded that its reaction with soluble SH components of the cell may also play a part in inhibition. In suggesting later a theory to explain the apparent Lundsgaard effect, we shall not therefore support the opinion offered by Nilson, Zeile & Euler, that iodoacetate acts on a later stage in fermentation. We shall assume that it acts on triosis.

IV. THE ESTABLISHMENT OF THE "LUNDSGAARD EFFECT"

We have shown that Lundsgaard was probably justified in his belief that fermentation inhibition by iodoacetate is due to the direct inhibition of triosis. We may now examine his statement ((3), p. 149) that certain differentiating concentrations of iodoacetate inhibit the fermentation in a sample of yeast, while having no effect on the rate of oxygen uptake in a comparable sample. We believe that he is justified in a modified statement of this kind, which may be put: in comparable samples of plant tissue, iodoacetate acts more rapidly in depressing the rate of fermentation than it does in depressing the rate of oxygen uptake associated with true carbohydrate respiration. In this section we shall briefly present the evidence for the modified statement, and we shall examine its consequences in Section V.

It is now clear, following papers by various authors, that it is comparatively easy to establish the existence of an apparently very sharp differentiation in the effect of iodoacetate on respiration and fermentation. Thus Boysen-Jensen (1931) showed that the rate of oxygen uptake in a sample of yeast was only slightly depressed by iodoacetate at a concentration of 1/5000 ($M^{-3} \times 1{\cdot}08$), while the same poisoned material, placed later in nitrogen, ceased to ferment after a few hours. He thus demonstrated that oxygen does not destroy the iodoacetate; such destruction, if it occurred, would render a simple explanation of the Lundsgaard effect possible. Boysen-Jensen failed to consider, however, that an inactivation of iodoacetate by oxygen might be a reversible process. If such reversible inactivation of iodoacetate could be demonstrated, the Lundsgaard effect would be explicable on the Blackman hypothesis.

Genevois and his collaborators (1933) have also stated that iodoacetate, bromacetate, and several derivatives of these compounds suppress yeast fermentation but have little effect on the rate of

oxygen uptake. They believe this to be a further demonstration of the thesis put forward by Genevois (1928), in a series of papers on the respiration of the green algae. We quote Cayrol and Genevois (1931):

> La fermentation et la respiration sont donc deux phénomènes entièrement distincts, susceptibles de varier entre de très larges limites indépendamment l'un de l'autre: KCN peut inhiber totalement la respiration sans agir sur l'intensité propre de fermentation; les acides acétiques halogénés inhibent totalement la fermentation sans faire varier l'intensité respiratoire.

The work of these French authors concerning iodoacetate has been published so far in the form of short notes without sufficient data to support their rather emphatic statements. That the matter is more complex than they would make it appear will be shown when we come to the later work of Lundsgaard (1932) and the present author.

In two more very brief notes, Boysen-Jensen (1931) and Radoeff (1933) have suggested that the Lundsgaard effect is obtained for the tissues of the higher plants. Boysen-Jensen soaked the cotyledons of peas in a solution of iodoacetate and showed that the ratio of the fermentation rate to that of the oxygen uptake was reduced. Radoeff too, stated that monobromacetate reduced fermentation (F) in peas while allowing normal respiration to go on. He also stated that bromacetate of ethylglycol reduced the rate of fermentation in wheat seeds without altering their capacity for growth in oxygen.

Nilson *et al.* (1931) examined the subject more thoroughly working with yeast; they showed that the range of differentiating concentrations was a very narrow one. All concentrations of iodoacetate between $M/100$ and $M/2000$ inhibited both F and R at pH 6·4. Concentrations near $M/10{,}000$ inhibited only F, not R, while those weaker than this had no effect on respiration (R) and only partially inhibited F. These results were later confirmed by Lundsgaard (1932), who showed that the pH of the medium was also an important factor. The rate of inhibition of both F and R in living yeast varied markedly with the pH of the buffer in which the iodoacetate was dissolved. This is well shown in the curves published by Lundsgaard. A concentration of iodoacetate which inhibited F after 75 min. at pH 5·0 had no effect at pH 7·0 after the same time. This concentration also depressed the oxygen uptake in a comparable sample by 75 per cent at pH 4·0, after 75 min., but had no effect in this time at pH 5·0.

These workers all used the rate of oxygen uptake as an index of the rate of carbohydrate respiration. In a more recent paper by

Lundsgaard (1932), however, it has been clearly shown that yeast poisoned with iodoacetate may show an uptake of oxygen not associated with a parallel oxidation of carbohydrate. Lundsgaard noticed that the longer the period of initial fermentation (Angärung) of the yeast before the application of iodoacetate, the better the differentiation obtained in the effect on *F* and oxygen uptake. He found that much of the oxygen uptake in yeast poisoned with iodoacetate was associated with an oxidation of alcohol. He therefore admitted that the greater part of the oxygen uptake going on in yeast poisoned with a "differentiating" dose of iodoacetate was due to alcohol oxidation, not to true respiration. He has even stated: "In einigen Versuchen habe ich versucht, die Hefe sofort nach der Angärung auszuwaschen, um eine Beeinflussung der R.Q. der erster Versuchsperiode durch Alkohol aus der Angärungszeit zu verhüten. Es hat sich indessen erwiesen, dass die Respiration in derart behandelten und danach vergifteten Proben so stark leidet, dass dieses Verfahren nicht anwendbar ist."

Ehrenfest (1932, 1933) has confirmed these results of Lundsgaard, and has shown that yeast can oxidize alcohol in the presence of concentrations of iodoacetate sufficient to inhibit both *F* and *R*. She follows this up by stating explicitly that iodoacetate inhibits true carbohydrate oxidation at the same rate, and to the same extent as the fermentation. She therefore believes that the Lundsgaard effect is only apparent and that the whole of the oxygen uptake taking place in the presence of the so-called differentiating dose of iodoacetate is due to alcohol oxidation only.

On the other hand, Lundsgaard (1932) still holds to his original view in essentials, and states his belief that some carbohydrate oxidation still goes on in yeast poisoned with iodoacetate at a concentration sufficiently great to inhibit fermentation completely. This view is to some extent confirmed by work carried out by the present author.

As reported in full elsewhere (Turner, 1937), he has carried out a series of experiments with excised disks of carrot tissue. The R.Q. of this tissue in water or buffer solutions is only very slightly above 1·00, and the oxygen uptake is a true index of respiration. It is therefore possible, using manometric methods (1930), or the continuous current method, to examine the effect of iodoacetate on respiration in the absence of appreciable quantities of alcohol.

In such experiments it has been shown that the fermentation of carrot tissue in nitrogen is inhibited by iodoacetate, though more

slowly than is the fermentation of yeast. In many experiments with aerated tissue in water, it was found that the iodoacetate also reduced the rate of respiration, as measured by the carbon dioxide output and the oxygen uptake. These experiments indicated, however, that the effect of a given concentration of iodoacetate on respiration, although progressive, was less marked than it was upon the fermentation, that is, the "modified" Lundsgaard effect was obtained (see p. 156, l. 17). It has not so far been possible to make the differentiating effect more marked by changing the *p*H of the medium.

However, in some experiments in which the disks were placed in glucose solution a more clearly marked Lundsgaard effect was obtained. In one experiment, complete differentiation was shown: the iodoacetate had no effect on the rate of oxygen uptake over a period of several hours, whereas the same tissue almost ceased to ferment after a further 9 hours in nitrogen. But in this experiment, as in those with yeast, in which the same effect has often been obtained, we cannot be certain that the carbon dioxide output was all associated with carbohydrate respiration. The R.Q. of carrot disks in glucose solutions is 1·08 and alcohol is produced. Moreover, the author has shown that the carrot tissue resembles yeast in that while poisoned with iodoacetate it can oxidize alcohol. (Alcohol increases the oxygen uptake of such tissue and reduces the R.Q.) It therefore seems that we may safely generalize by stating that iodoacetate, at a concentration just sufficient to inhibit F, probably always reduces the rate of true respiration. At any rate, it has not yet been shown in the numerous experiments with both yeast and carrot tissue, that such a concentration has absolutely no effect on respiration. It is possible, of course, that future work, in which the effect of alcohol is taken into full consideration, will demonstrate a complete differentiation: but until such a result is obtained we feel justified in concluding that iodoacetate has essentially the same inhibitory effect on both fermentation and respiration, but that it acts more quickly on fermentation than on respiration.

V. An interpretation of the "Lundsgaard effect"

A certain differentiating concentration (D) of iodoacetate is applied to a sample of yeast; if the sample is placed in nitrogen its fermentation ceases after about 1 hour; if in oxygen the true respiration continues, though at a reduced rate, for several hours. The same, in general, holds for carrot tissue. Lundsgaard believes that in the

aerated sample the enzymes of F are inhibited, and that the respiration going on is at the expense of carbohydrate broken down in some way which does not involve triosis. He then concludes that in normal tissues also, triosis and respiration are unconnected.

He thus treats the yeast cell merely as a complex of enzymes, and makes the assumption that any external concentration of iodoacetate bears to the "internal effective" concentration the same relation in oxygen as in nitrogen.

The present author considers that this is an unjustified assumption and offers evidence which suggests, though it does not prove, that for a given external concentration the internal effective strength of iodoacetate is less in oxygen than in nitrogen. Thus his explanation of the above type of experiment is that iodoacetate inhibits both respiration and fermentation by inhibiting triosis, but that in oxygen the added concentration (D) is less effective as an inhibitor of triosis than it is in nitrogen. Two pieces of evidence may be offered in support of this view.

(1) The rate of inhibition of fermentation is no greater when the iodoacetate is added to the fermenting tissue than when it is added previously in oxygen.

Suppose the concentration D of iodoacetate causes complete inhibition of fermentation in (t) hours, and let this concentration be applied to aerated tissue for this length of time. The rate of respiration will not be zero after time (t), as (D) is supposed to be the differentiating concentration. According to Lundsgaard, however, the iodoacetate should have inhibited triosis completely in (t) hours, and on transference of the tissue to nitrogen we should find zero fermentation. But experiments with carrot tissue have shown that after such transference of pre-treated tissue, there is an appreciable though falling rate of fermentation over a period of the order of (t) hours. The time required for a 50 per cent inhibition of the fermentation when the iodoacetate is added in nitrogen is not significantly greater than that required when the iodoacetate is added some hours previously in air, and the system then placed in nitrogen.

It is therefore suggested that the effective strength of the iodoacetate is increased when the tissues are transferred from aerobic to anaerobic conditions.

(2) The more convincing line of evidence is provided by two experiments in which was measured the direct effect of oxygen on the fermentation inhibition by iodoacetate. It was clearly shown that the rate of fermentation inhibition by iodoacetate is decreased by the presence of small amounts of oxygen.

The rate of fermentation in carrot tissue is not greatly reduced by the presence of 2·5 per cent by volume of oxygen. It is therefore possible to compare the rates of fermentation inhibition by a concentration (D) of iodoacetate, in pure nitrogen, and in 2·5 per cent oxygen. The percentage rates of inhibition are calculated from appropriate controls in manometric experiments. In two experiments with iodoacetate at 1/40,000 ($1{\cdot}34 \times M^{-4}$), fermentation in pure nitrogen was completely suppressed after 5–6 hours. The fermentation rate in 2·5 per cent oxygen was more slowly reduced, and after 8 hours the percentage inhibition was not more than 95 per cent. The half times for the inhibitions in nitrogen were 2·1 and 2·9, while in 2·5 per cent oxygen they were 3·8 and 4·1. The difference between the rate of inhibition in nitrogen and in 2·5 per cent oxygen is of the order of that found between the rates of inhibition of fermentation and respiration caused by one concentration of iodoacetate.

Two possible explanations of the effect of oxygen can be advanced. Oxygen may decrease the permeability of the cells to iodoacetate. Some evidence has already been mentioned which shows that the alteration of the cell permeability by *p*H changes may affect the rate of fermentation inhibition, but so far we have no direct evidence as to a similar effect of oxygen.

Alternatively we may suppose that iodoacetate reacts with or combines with some substance in the cell before causing inhibition of triosis: and that this reaction takes place more slowly in the oxygenated than in the anaerobic cell. As a model of such a system we will suppose that iodoacetate, in causing inhibition, reacts with a reduced sulphydryl radicle, and that the equilibrium:

$$\mathrm{SH} \rightleftharpoons \mathrm{SS}$$

within the cell is affected by oxygen. A shift of the equilibrium to the right in the aerated cell might then slow down the rate of reaction of the iodoacetate with the SH radicle. Although, as shown in p. 152 we no longer take the view that glutathione itself plays a part in the inhibition of triosis by iodoacetate, there is nevertheless the possibility that other SH radicles, notably those of the proteins, are involved. Moreover, there has recently been a tendency on the part of biochemists to regard a shift in the equilibrium of the SS $\rightleftharpoons$ SH reactions as of importance in the regulation of metabolism.

In support of his explanation of the Lundsgaard effect, the writer can therefore offer direct evidence, and also a plausible picture of the mechanism involved. It is to be noted in this respect that Lundsgaard has as yet offered no explanation of the depression of the

respiration by iodoacetate, although this is now generally admitted as fact.

VI. THE RESPIRATORY QUOTIENT OF TISSUES POISONED WITH SODIUM MONOIODOACETATE

The respiratory quotient in normal yeast approximates to 1·4. This is due to the existence of some aerobic fermentation, which results in the production of alcohol and carbon dioxide without a corresponding uptake of oxygen. On the Lundsgaard hypothesis, this means that the rate of triosis is greater than that of the Meyerhof reaction; and on the Pfeffer-Blackman hypothesis, that the rate of triosis is greater than the combined rates of oxidation and oxidative anabolism.

In his first paper Lundsgaard stated that the R.Q. of yeast, poisoned with iodoacetate so that its rate of respiration was 40 per cent of the normal, was 1·00. Cayrol & Genevois (1931) confirm this with a single protocol. Lundsgaard (1930, 1930*a*) thought that the low R.Q. indicated the complete inhibition of aerobic fermentation at a concentration which did not completely inhibit respiration. He therefore claimed that the lowering of the R.Q. was an important link in the chain of evidence in favour of his hypothesis.

In later and more critical work, however, Lundsgaard (1932) showed that iodoacetate-poisoned yeast may oxidize alcohol with a R.Q. of only about 0·4, organic acids being formed. Moreover, aerobic yeast produces alcohol. More careful measurements of the R.Q. of yeast in iodoacetate showed that during the first hour of poisoning, the R.Q. was about 0·8, and that it subsequently rose. Lundsgaard stated that the average R.Q. over several hours of poisoning was 1·00, and he therefore believed that these results confirmed his earlier work. His actual figures show, however, that after the first hour the R.Q. rises to values near 1·1 or 1·15, though never reaching the high values of normal yeast. It is therefore a coincidence that in his rather short experiments the average R.Q.'s, which include the initial low values, are so close to 1·00.

Lundsgaard's interpretation of his results is that, during the first hour of poisoning, the yeast is oxidizing the alcohol formed during Angärung; and that when all this has gone, the R.Q. is 1·00, and no more alcohol is formed, indicating the complete inhibition of aerobic fermentation and the existence of true carbohydrate oxidation in the presence of iodoacetate. The writer offers another explanation more in keeping with the actual figures quoted above. The existence of a

R.Q. above 1·00 after the first hour suggests that aerobic fermentation is still proceeding in the poisoned yeast. If so, then alcohol is being produced, and some of it is presumably oxidized with a low R.Q. of about 0·4. Thus, even after the complete oxidation of the alcohol of Angärung, the R.Q. measured may be due to a combination of a high R.Q. due to aerobic fermentation, and a low R.Q. due to alcohol oxidation. The net R.Q. might be 1·00: when, as here, it is greater than 1·00, we may postulate quite a considerable aerobic fermentation in the presence of the iodoacetate. It is admitted then that some true carbohydrate respiration may be going on in the poisoned yeast, but it is suggested that the lowering of the R.Q. may be due, partially at least, to some alcohol oxidation, coupled with only a partial inhibition of aerobic fermentation.

But even if it had been shown that there was no alcohol oxidation in poisoned yeast after the first hour, and that the R.Q. was then really 1·00, this would not necessarily mean the abandonment of the Blackman hypothesis. For on this hypothesis, the aerobic fermentation in yeast is associated with a high rate of triosis, some of the products of which appear as fermentation end products. If the affinity of oxygen for these products is high, the lowering of the rate of triosis by iodoacetate applied in air would lead first to the cessation of the formation of fermentation end products, and the lowering of the R.Q. at 1·00. On this view the rate of respiration would be maintained at the normal until the R.Q. had fallen to 1·00, and it would only then begin to fall. And again, this fall might be masked by alcohol oxidation.

Experiments with carrot tissue have on the whole confirmed those of Lundsgaard with yeast, and they may be explained in the same way. When alcohol is added to tissue poisoned with iodoacetate, in which the rate of *R* is low, the rate of oxygen uptake is increased, and the R.Q. falls below 1·00. Again, iodoacetate reduces the R.Q. of carrot tissue in glucose from 1·08 to 1·00.

By lowering the partial pressure of oxygen over carrot tissues the metabolism may be made to simulate that of yeast. That is, under these conditions the tissue both respires and ferments, and the R.Q. is raised above 1·00. Thus the R.Q. of carrot tissue in glucose solution in equilibrium with 5 per cent oxygen was 1·7. The relatively strong iodoacetate solution of 1/50,000 lowered the R.Q. of such tissue to 1·1, but had no appreciable effect on the rate of oxygen uptake over several hours. A stronger concentration of 1/10,000 lowered the R.Q. to 0·7, and caused a slow progressive diminution in the rate of

oxygen uptake. The experiment thus confirms those on yeast, and also strongly suggests that the Lundsgaard effect is more clearly shown by tissues whose R.Q. is high, and which produce alcohol even in oxygen. Explanations of this have already been put forward.

VII. Respiration after fermentation inhibition caused by sodium monoiodoacetate

Further experiment is obviously required to decide between the two explanations of the Lundsgaard effect. In principle the method is simple. It is to discover whether tissue, whose fermentation in nitrogen has been completely inhibited by iodoacetate, carries out any oxidation of carbohydrate (respiration) on transference to aerobic conditions. If we can demonstrate such an after effect and couple with it a proof that fermentation inhibition is not reversed by oxygen, we shall be justified in assuming that the tissue is capable of oxidizing sugars without triosis. It would not, however, necessarily follow that in normal cells the products of triosis were not oxidized.

In his first papers Lundsgaard (1930, 1930*a*) stated that yeast could take up oxygen subsequently to the inhibition of fermentation by iodoacetate. Boysen-Jensen (1931) confirmed this. Their few published data show low but rising rates of oxygen uptake for at least 2 hours, by yeast returned to oxygen following complete fermentation inhibition. Experiments by the writer have also convinced him that it is comparatively easy to obtain such apparent recovery of the respiration in yeast.

But it is now realized that the yeast under such conditions may be merely oxidizing alcohol or some other intermediate accumulated in the cells during the preceding fermentation. In fact, Lundsgaard has himself demonstrated that alcohol oxidation is going on in the period of apparent recovery. No data so far published for yeast have demonstrated conclusively that true carbohydrate respiration is going on in the period of the "after effect". In fact, Ehrenfest (1932) has stated categorically:

> When fermentation is completely inhibited in yeast by iodoacetate, no oxidation of glucose occurs, although lactate, pyruvate, and alcohol are still oxidized at rates lower than those in the absence of oxygen.

It is much to be desired that the evidence on which this statement is based should be published in greater detail, and the results confirmed.

A number of experiments with carrot tissue have been made to try to settle this point, but they were not conclusive. In those in

which the iodoacetate was added to the fermenting cells at a concentration greater than the "differentiating" one, no recovery of carbon dioxide output or oxygen uptake was obtained, as one would expect from the knowledge that such concentrations inhibit respiration when applied directly to aerobic tissues.

In four experiments with the differentiating concentration, well marked "after effect" was obtained. The iodoacetate applied in nitrogen reduced the carbon dioxide output to a low value, about 10 per cent of the normal fermentation rate. When air was passed the rate of carbon dioxide output rose from this low value to a peak which was never more than 60 per cent of the normal respiration rate. The carbon dioxide production then fell off again as rapidly as it had risen, reaching a rate equal to that of the depressed normal respiration rate after about 20 hours from the transference to air.

These curves, while hardly suggesting a true recovery of a normal respiration (their shape rather suggests the oxidation of some accumulated intermediate) nevertheless meant that the poisoned tissue was evolving a considerable quantity of carbon dioxide following an almost complete inhibition of fermentation. Moreover, it can be calculated that even if all the alcohol produced in the previous period of fermentation is oxidized during the aerobic period with an R.Q. of 0·4, this will only account for about half of the carbon dioxide evolved. The experiment would therefore tell against the writer's hypothesis if it were not that the fermentation inhibition in these experiments is never complete. We have, in fact, some cells or parts of cells (probably those cells in the interior of each disk) still carrying out triosis at the transference to air, and it may well be that the carbon dioxide not accounted for by alcohol oxidation is evolved from these cells. At least until we have proof that this is not so the author is not inclined to use these experiments in support of Lundsgaard's claims, in view of the evidence which he has already presented. It may be pointed out that at weak concentrations of iodoacetate many hours are required to obtain complete fermentation inhibition, and that owing to the danger of bacterial infection in long experiments it is not practicable to wait so long before transference to air.

An experiment is therefore required in which the fermentation is completely inhibited, any alcohol removed or accounted for, and the rate of true carbohydrate respiration measured followed aeration. No such experiment has yet been carried out. Even if under these

conditions true recovery of respiration were demonstrated, we should still have to consider a statement of Ehrenfest's (1933):

> A period of alcohol oxidation by poisoned yeast restores the power of fermentation to cells whose fermentation has been inhibited by iodoacetate.

This statement has been made without a clear account of the experiments leading to it. The present writer is endeavouring to confirm it, but so far has had no success.

VIII. SUMMARY

1. The two opposed theories of the relation between the respiration and fermentation are discussed, and it is indicated how work with iodoacetate may enable one to discriminate between them.

2. The views of Lundsgaard, as based on early work with iodoacetate, are set out in full.

3. It is shown, after a review of recent evidence, that Lundsgaard was probably justified in concluding that iodoacetate inhibits fermentation by direct action on the first stage of fermentation, here defined as triosis.

4. Recent evidence indicates that iodoacetate inhibits fermentation more rapidly than oxidation; the earlier statements, that differentiating concentrations of iodoacetate did not affect respiration were due to a neglect of the alcohol oxidation going on in poisoned tissue.

5. An explanation alternative to that of Lundsgaard is offered to account for the differentiation in the effect on respiration and fermentation. Evidence is presented to show that the internal effective concentration of iodoacetate is probably smaller when the cells are aerobic than when they are in nitrogen. The author is therefore of the opinion that iodoacetate, by inhibiting triosis, inhibits both fermentation and respiration. He believes that as far as work with iodoacetate goes, the Blackman hypothesis still holds good.

6. Further evidence with respect to the R.Q., and to the respiration following fermentation inhibition is discussed, and it is concluded that until certain features in this evidence are clarified, it cannot be used to determine which of the explanations of the Lundsgaard effect is the right one.

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THE RESPIRATION OF BANANAS IN PRESENCE OF ETHYLENE

By R. GANE, M.Sc., Ph.D.
Low Temperature Research Station, Cambridge

(With 7 figures in the text)

The banana fruit of commerce is always gathered immature and green and ripened after transport. There are not yet available the results of any complete study of the temperature-respiratory activity-time relationships of immaturely gathered fruit. Such results as have been obtained with post-transport fruit (1936) suggest that the onset of ripening is accompanied by a sharp rise in respiratory activity similar in general character to that occurring in the apple

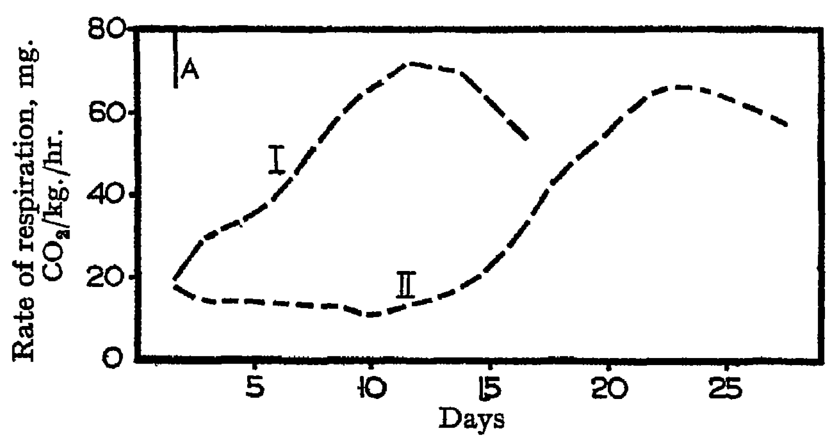

Fig. 1. Rate of production of carbon dioxide by bananas at 15° C. in air and in air containing 1 part per million of ethylene. Bananas II in fresh air throughout. At A, bananas I ventilated with air containing 1 p.p.m. of ethylene.

and pear as described by Kidd & West and termed by them the climacteric. In the case of the apple these authors have shown that ethylene administered before the climacteric stimulates its immediate onset and that administration after the climacteric has no effect.

A series of experiments has been carried out in which post-shipment bananas have been treated with ethylene in the pre-climacteric phase of low respiratory activity. In all cases the action of ethylene has been the stimulation of the immediate onset of the rise in respiratory activity associated with ripening.

Fig. 1 presents the results of one such experiment. Comparable samples of fruit were obtained by using the two halves of a hand, one

of which was ventilated with air and the other with the gas mixture. The gas mixture was made by introducing 2·1 c.c. of ethylene in a partially evacuated steel cylinder and then adding 75 cu. ft. of air from a similar cylinder containing 150 cu. ft. of air under pressure.

Fig. 2 presents the result of an experiment in which ethylene was administered in the post-climacteric phase. At this stage it is without effect on respiratory activity.

An interesting extension of this type of experiment is one in which the application of ethylene is made after the upward tendency in respiratory activity has already begun. The results obtained in this case are shown in Fig. 3. It will be seen that the rate of rise is accelerated.

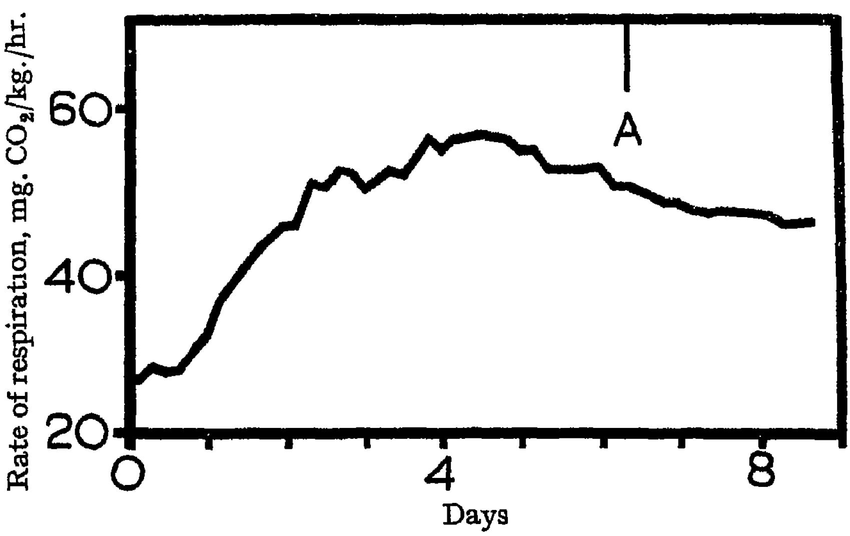

Fig. 2. Rate of production of carbon dioxide by bananas at 15° C., in air and in air containing ethylene At A, 1 c.c of ethylene was added to the air stream.

Now in the case of apples the present author (1935) has shown that with the onset of the climacteric rise in respiratory activity ethylene is produced by the fruit itself, while Kidd & West have shown that the volatiles from post-climacteric apples stimulate the climacteric in unripe fruit and also that the same is true in the case of bananas. Confirmation of their observation with regard to bananas has been obtained.

Three samples of bananas were obtained from one hand of fruit, sample I was ventilated with air from a cylinder, sample II was similarly ventilated but 0·1 c.c. of ethylene added to the air stream, while sample III was ventilated with air that had previously been used to ventilate ripe bananas and then passed over moist soda lime to remove carbon dioxide.

The course of respiratory activity of all three samples is shown in Fig. 4. Readings of the respiratory activity were obtained every

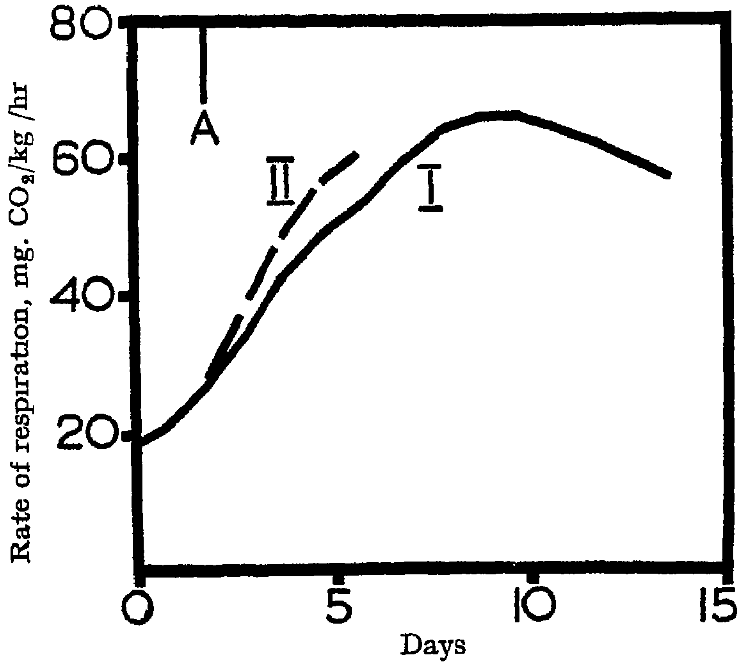

Fig. 3. Rate of production of carbon dioxide by bananas at 15° C., in air and in air containing 1 part per million of ethylene Bananas I in air throughout. At A, bananas II ventilated with air containing 1 p.p.m. of ethylene.

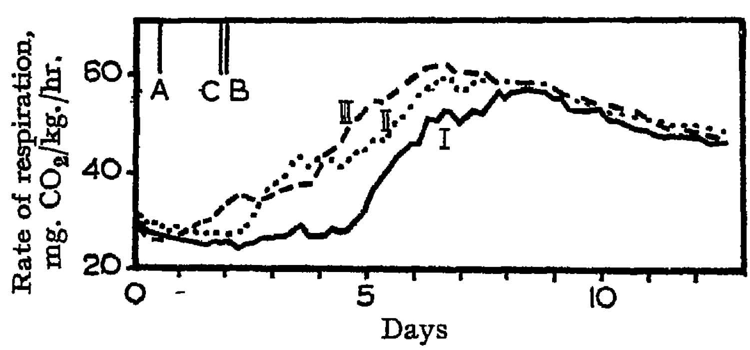

Fig. 4. Effect of volatile products from ripe bananas on the production of carbon dioxide by unripe bananas (at 15° C.). Banana I in fresh air throughout. At A, air from a ripe banana was passed over banana III. At B, banana III was returned to fresh air. At C, 0·1 c.c. of ethylene was added to air stream over banana II.

4 hours, and it will be seen that in samples II and III the respiration started to increase after 8 hours from the time dosing started.[1]

A germinating-seed test for the production of traces of ethylene by bananas has been applied. Peas germinating in a closed vessel in

[1] In this particular series, the peak of respiratory activity was reached at almost the same time by all three samples, and although the respiration was increased by the treatment it was observed that ripening in this experiment did not appear to be greatly accelerated by the ethylene treatment.

the presence of ripe bananas at 25° C. with the minimum ventilation required to keep the carbon dioxide below 10 per cent exhibited the abnormalities in growth which are described as typical of the effects of ethylene (Crocker, 1929). Such effects, however, were only produced by restricting ventilation and at a high temperature. Similar effects are produced by apples with ventilation rapid enough to keep the carbon dioxide below 0·5 per cent and at lower temperatures. When unripe green bananas are used these effects are not produced.

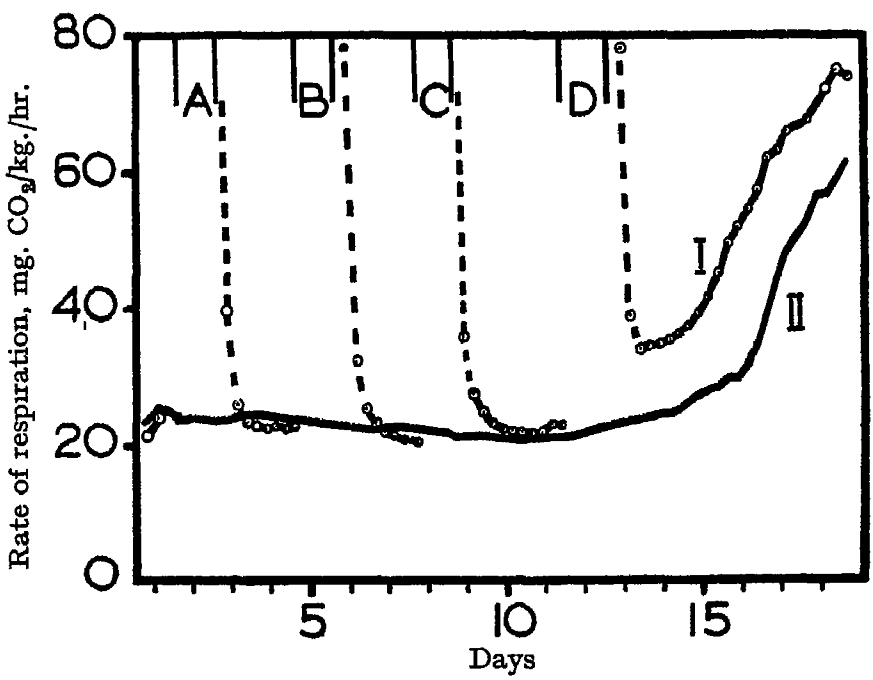

Fig. 5. Production of carbon dioxide by bananas in air at 15° C. with continuous and discontinuous ventilation. Bananas II, continuous ventilation throughout. Bananas I, no ventilation at periods A, B, C and D.

In parallel with these germinating-seed tests for the presence of ethylene, the effects upon the subsequent respiratory activity of bananas were studied after periods in which ventilation was restricted to allow the accumulation of 10 per cent carbon dioxide. The data shown in Fig. 5 refer to two samples from the same hand of fruit, one sample was ventilated continuously and the other discontinuously.[1]

There is no sign of the action of any stimulating substance in the pre-climacteric phase, but as soon as the fruit passes into the climacteric phase, a period of restricted ventilation accelerates the completion of the climacteric.

There is a strong probability therefore that bananas behave like apples and begin to produce ethylene with the onset of the climacteric.

[1] There is, of course, an initial high value due to the accumulated carbon dioxide, but this is soon lost.

The fact that treatment of the fruit with ethylene (1 part per million) or a temporary restriction of ventilation *accelerates* the climacteric rise is interesting. In their work on apples and pears Kidd & West (1933) have shown on the one hand that restricted ventilation or ethylene treatment will stimulate a climacteric change even in very immature fruit, and on the other hand that if single fruits are used (pears in this case), gathered sufficiently immature and ventilated sufficiently rapidly, no climacteric ever occurred. They argue that (1) ethylene or a substance with similar action is being produced prior to the climacteric; (2) there must be a threshold value of ethylene concentration in the cells necessary for stimulation; (3) probably the rate of production of ethylene rises with age while the threshold value for stimulation falls. The results of the experiments described above suggest that the cells of the fruit do not all reach the point of auto-stimulation simultaneously. If so, it should be expected that at the stage when some have reached the critical point and others are near it, an artificial raising of the concentration of ethylene would cause an accelerated rise in the respiratory activity of the whole fruit.

The active substance from ripe bananas is destroyed, or inactivated, by ozone. A stream of air was passed over *ripe* bananas, and then divided into two halves, each of which was led over a similar sample of green bananas; into one stream a small oxonizer introduced ozone at a concentration of from 70 to 90 parts per million. The bananas exposed to this gas ripened at a much later date than the other sample.

In this experiment the lenticels of the fruit exposed to ozone became discoloured and dark green unfiltrated areas were formed round them, which later became brown, and finally black. The experiment was repeated in a modified form in which the comparison instituted was between fruit ventilated with fresh air in the normal way and fruit ventilated with air passed first over ripe bananas, then through an ozonizer, and finally through a deozonizer.[1] The behaviour of the two samples was identical. The ozone had clearly removed stimulating substances while the deozonizer had eliminated the damaging effects of ozone.

With a view to surveying the sensitivity of the banana fruit to ozone comparable samples of bananas were stored at 15° C. and ventilated with air and with air containing a low concentration of ozone. The ozone was produced by a small ozonizer built into the

[1] A glass tube filled with scrap rubber.

inlet tube of the fruit container. The quantity of ozone produced could be varied by altering the resistance in the mains leads of the high tension transformer. The concentration of ozone is approximately constant over a range of air speeds. Any ozone in the air stream issuing from the fruit container was removed by passing through a glass tube containing scrap rubber.

Even the lowest concentration of ozone used, 1·5 parts per million, caused injury to the peel. A new observation was also made, namely, that one of the effects of ozone is a retardation of ripening. This retardation is probably due to a plugging of the stomata of the fruit by the decomposition products formed by the action of ozone. The data are plotted in Fig. 6. A similar retardation of ripening is shown by fruit treated with hydrogen peroxide, dilute aqueous iodine and coated with vaseline, and the conclusion has been drawn that the effect is due to a blocking of the channels through which the gaseous exchange takes place and consequently to an increased carbon dioxide and diminished oxygen tension within the fruit.

Certain suggestions of possible value in the handling of fruit arise from the facts established above. The first is that the problem of "ships ripes" in banana-carrying vessels may find its partial solution in the combined use of ozonizers and deozonizers for the treatment of the air circulated through the holds and over the coolers by fans. The second is that great care should be taken to avoid storing ripe fruit or fruit that has been treated with ethylene, as is often the case with early gathered oranges, in the same air with unripe fruit. The effect upon bananas of the volatile products of a ripe apple (Bramley Seedling) is shown in Fig. 7. Sufficient ethylene escapes from one apple ventilated at the rate of 25 c.c. per min. to start the ripening processes of bananas immediately. In view of the work of Kidd & West (1932) on the ripening of apples, it is probable that premature ripening of bananas also can be induced by the volatile products from pears, peaches and tomatoes, but not by fully coloured oranges or grapes.

The opinion is held in the banana-carrying trade that oranges and citrus fruits in general are potential sources of danger and liable to cause the bananas to ripen. Laboratory experiments do not confirm this, though it should be remembered that, when citrus fruits have been treated with ethylene, this gas would dissolve in the tissues of the fruit and escape from solution again after the fruit was removed from the conditioning rooms. There is no evidence as to the time required for all the ethylene to be lost from these tissues.

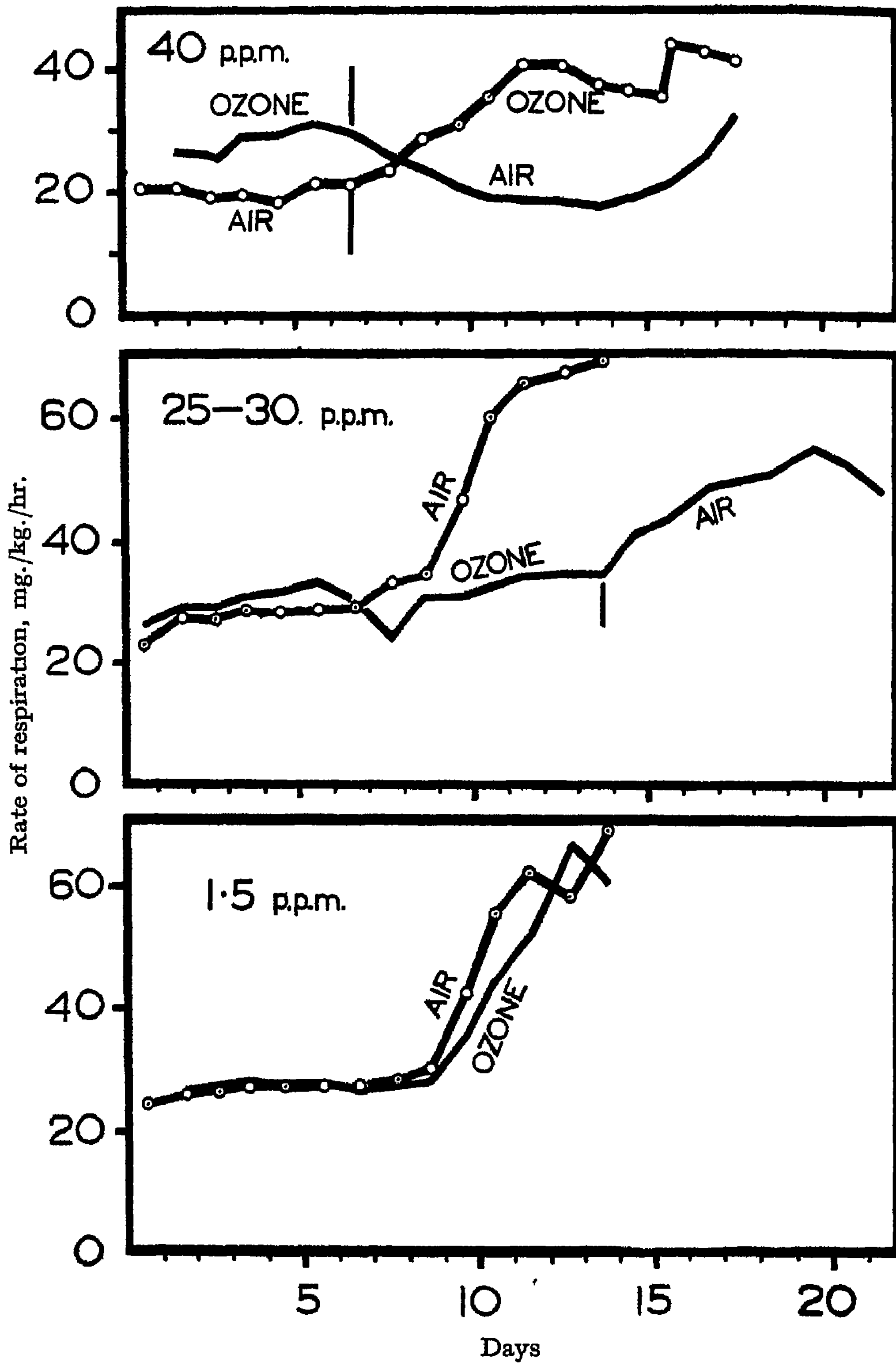

Fig. 6. The effect of ozone on the production of carbon dioxide by bananas at 15° C.

Laboratory tests suggest that no ethylene-like substance is produced by rotting banana stems, rotting bananas, yeast growing in a culture solution, or by onions. None of these produce any substance which acclerates the ripening of bananas. Using a different indicator, either tomato shoots or potato shoots, Denny, Miller, Crocker,

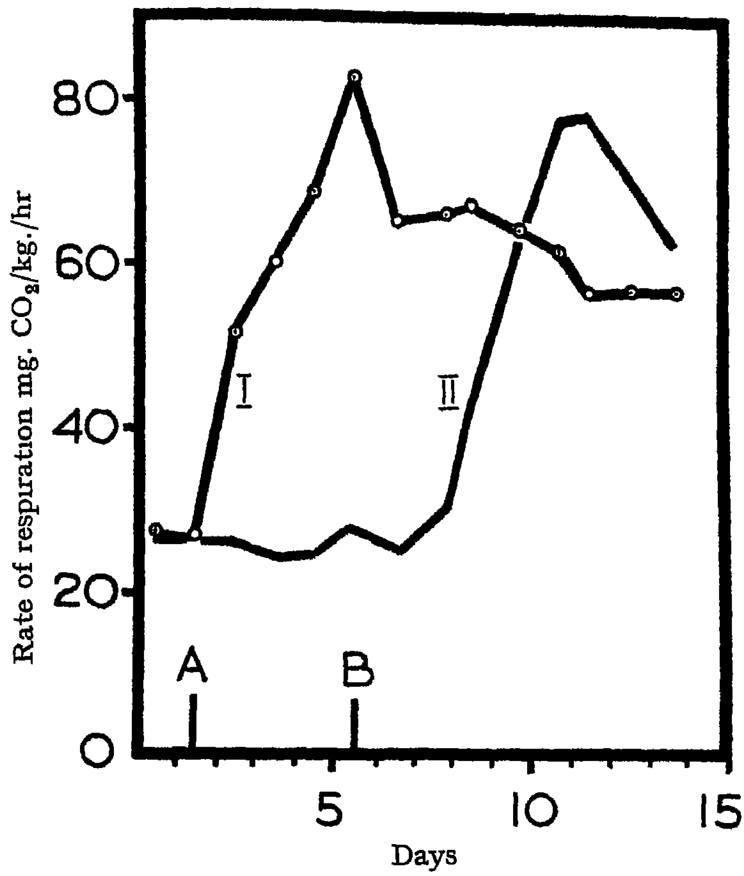

Fig. 7. Effect of the volatile products from an apple on the production of carbon dioxide by bananas at 15° C. Bananas II in fresh air throughout. At A, air was passed over an apple (Bramley Seedling) and then over bananas I. At B the apple was removed from the air stream.

Hitchcock & Zimmerman (1935) have shown that ethylenc is produced by a variety of tissues including parts of flowers, e.g. petals, anthers; immature fruits; leaves; roots and tubers; but data is not available to show whether the quantity produced is sufficient to affect bananas.

SUMMARY

Evidence is given to show that ripe bananas produce ethylene. The effect on seedlings of *Pisum sativum* is paralleled by low concentrations of ethylene.

An acceleration of ripening of unripe bananas by ethylene at a concentration of 1 part per million is similar to that produced by

the products of metabolism of ripe bananas. It would appear that ethylene is a normal product of metabolism during the climacteric when it acts as an autocatalyst.

Ethylene can be removed from air by ozone.

Ozone causes a retardation of normal ripening, probably by gumming up the stomata. Similar retarding effects are shown by fruit treated with hydrogen peroxide, iodine and vaseline.

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A GENETICAL INTERPRETATION OF ALTERNATION OF GENERATIONS

By H. C. PINCHER, B.Sc.
Liverpool Institute

A REVIEW of the literature dealing with this fascinating subject would be superfluous here, primarily because excellent summaries have been provided by others (e.g. Bower, 1935), and secondarily because this interpretation is based upon no pre-existing theories.

There has been much controversy with respect to the adequacy of cytological data as criteria of the generations. If it be supposed as a hypothesis that the diploid number of chromosomes is the fundamental character of the sporophyte, then surely the invalidity of such a hypothesis would be demonstrated by the existence of haploid sporophytes. Such haploid sporophytes and similarly diploid gametophytes are of course common, and it is therefore necessary at least to realize the limitations of cytological data as criteria of the generations.

It is important to understand what is really implied by the symbol *n*. Terminologically it symbolizes a certain number of chromosomes, but fundamentally it implies a number of genes, and cytogenetical evidence indicates that these are responsible for the production of all the characters of an organism, though their effect may be modified with respect to the environment in which they react.

In a plant such as a moss the gametophyte possesses certain characters which are absent in the sporophyte and vice versa. Since each character is but the effect of a gene or genes, it would appear that each generation possesses certain genes which are absent in the other. However in the self-fertilization of a pure line the diploid number of chromosomes implies only the duplication of genes and not the inclusion of new ones, so that all the characters both of the sporophyte and gametophyte are represented by genes included in the symbol *n*. It is important to grasp this fact, since some workers have unconsciously tended to regard the two generations as separate entities rather than developmental phases of a single one. The haploid complement of genes thus comprises certain units which produce their effect early, i.e. in the gametophyte and others which

react later, i.e. in the sporophyte. In the normal development of any plant, the characters do not appear simultaneously or at random, but follow a definite sequence both in the time and mode of their appearance. This means that the genes responsible for the production of these characters each produce their effect at a certain stage in ontogeny, and since these respective times are inherited characters, it would appear that the time of initiation of the reaction of each gene is controlled by another gene or genes. This conception of a time factor has already been realized (Brambell, 1930) and it is not necessary to go on postulating genes *ad infinitum* since the multiple factor theory, discussed at length and substantiated by Sinnott & Dunn (1935), holds that most genes have many effects, and thus the genes may be so interrelated both with themselves and with the environment that they mutually control the times of their reaction, in addition to producing their normal characters. If this time factor is genic, and it certainly appears to be, simple mutation in the genome could cause alteration in the time of reaction of one or more of its constituent genes. Such a mutation would show itself as a character which appeared abnormally late or early in development.

There can be no doubt that haploidy was the primitive condition, diploidy arising only at the initiation of sexuality and being necessarily accompanied by meiosis. Algal types such as *Spirogyra*, where meiosis follows quickly, are generally held to be primitive, and the diplophase of such plants is usually described as insignificant or simple. These terms really imply that the diplophase *possesses few characters*. From this algal source, the mosses are supposed to continue the evolutionary sequence, being characterized by the retention and nourishment of the zygote by the gametophyte. An alga such as *Coleochaete* shows this retention in a primitive manner, and though the zygote is still of the *Spirogyra* type, being reduced at its first division (Allen, 1905) it provides a type from which higher forms could have been derived. The antithetic theory holds that the sporophyte of higher types arose by the elaboration of such a primitive zygote. What is implied by this much-used term "elaboration"? *It can only mean the introduction of new characters and this of course could only happen through the introduction of new genes.* It is well known, however, that genes do not arise *de novo*, and there is thus only one source from which the sporophyte could derive its genes, viz. the gametophyte. The sporophyte could thus evolve only by the reaction in the diplophase of genes which formerly produced their effect in the haplophase, and since the sporophyte is only a later stage in the ontogeny of the plant,

this result could be brought about as described above by simple mutation in the genes controlling the time of appearance of the characters.

As the sporophyte evolved by the introduction of characters, the gametophyte would correspondingly lose characters, i.e. *it would be reduced*. This reduction of the gametophyte which universally accompanies elaboration of the sporophyte has been explained as a response to drier land conditions, but this interpretation has been refuted by the discovery of very reduced gametophytes in some seaweeds. On the basis of random mutation suggested above, not only are such aquatic types explained, but are actually to be expected.

It is now well known that the inherited genes alone are insufficient to account for the characters produced, since the mode of expression of a gene can be greatly altered by a changing environment. When the genes which normally reacted in the gametophyte came to react in the sporophyte, they would find themselves in a very different environment, partly owing to the general disturbance in gene-reaction, and partly because of the semi-parasitic habit of the zygote, and the characters they produced would be proportionately modified. Such an initial change would be intensified by mutation coupled with natural selection, these evolutionary factors adapting the sporophyte to land conditions.

One of the first characters which passed over to the sporophyte was the production of asexual spores, so that this mode of evolution would be exceptionally rapid since the chances of mutation would be greatly enhanced by the many meioses occurring at sporogenesis.

One of the last to pass over was the production of root-like organs, and as soon as the sporophyte developed roots it became independent. Thus a simple mutation with respect to this character bridges the gap between the mosses and ferns. The intermediate type is probably represented in the fossils by *Sporogonites*.

In this mutational manner, so many characters became sporophytic that as we pass to types such as presented by the ferns, we find practically only the sex-characters remaining in the gametophyte. Even these ultimately pass over to the diplophase and the different ways in which the genes produce their effect there have been fully described in a previous paper (Pincher, 1935). Suffice it to mention here that on this basis heterospory, heterangy, monoecism and dioecism are all simply explicable and follow on in a sequence from the fern type of alternation.

The condition in a haplobiontic red alga is directly comparable with that described above for the green algae, since the zygote is reduced at its first division, typically dividing directly into four carpospores which reproduce the gametophyte. In the evolution of the diplobiontic type a failure of meiosis occurred so that the carpospores were diploid. This failure was apparently of the nature of a mutation since it became an inherited character of the plant, but it affected only the *time* of meiosis and not its effects. Since the zygote was not retained, and the environment was therefore in no way peculiar, the ordinary developmental sequence of the genes was maintained and the carpospores produced plants exactly like the gametophyte. The two generations, gametophytic and tetrasporic have been styled homologous, and this is fundamentally correct since their morphological characters are expressions of the same genome, which is the only basic definition of homology. The diploid tetrasporic plants arise from carpospores which are abnormally diploid. This condition is identical with that described by Strasburger for *Marsilia Drummondii* (cited by Darlington, 1932) and included by Darlington under the term "Diploid parthenogenesis". The apparent diplophase in the diplobiontic red algae is thus nothing more than a recurrent diploid gametophyte, the true sporophyte being the zygote which is in the same condition as that of the haplobiontic forms. It is therefore not in the least surprising that attempts to compare it with the antithetic diplophase of higher types have resulted in confusion! It will readily be realized that since the diplophase is but a diploid gametophyte and both phases develop under the same conditions, a mutation in either phase will appear in both, so that the two generations are always bound to remain identical. An objection may be raised with respect to the fact that since the tetrasporic plant is a gametophyte it should produce gametes. These are not to be expected, however, since the products of the meiosis are in every way normal, i.e. they are carpospores (≡tetraspores), and not gametes. Only the time of initiation of meiosis, not the result, is abnormal.

On the grounds presented above all sporadic and otherwise disturbing types of alternation are explicable. Thus *Cutleria* may be regarded as an antithetic type with a sporophyte which is not retained, whilst *Zanardinia* and *Dictyota* present the antithetic-cum-homologous condition found in the Rhodophyceae. *Fucus* would appear to be an extreme form in which all the characters including those of sex have passed over to the diplophase.

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PRE-ROMAN BEECH CHARCOAL IN SOUTH WALES: A PRELIMINARY NOTE

By H. A. HYDE

Department of Botany, National Museum of Wales

THE question whether beech ever occurred spontaneously in Britain has been much discussed of recent years and though discoveries of fossil pollen in Northumberland, the Fens, and elsewhere have seemed to provide an affirmative answer, satisfactory confirmation by finds of wood remains has heretofore been wanting. Such evidence has recently come into my hands. I have had an opportunity to examine a batch of charcoal fragments which formed part of the material excavated in 1914 from a so-called prehistoric hearth at Radyr, near Cardiff, Glam. Out of ninety such fragments, all of which were large enough (averaging an inch across) to allow of ready identification, twelve proved to be beech, a fact which Mr B. J. Rendle of the Forest Products Research Laboratory, Princes Risborough, very kindly confirmed for me.

The date of occupation of the site appears to be fixed within sufficiently narrow limits by the occurrence along with the charcoal of a number of potsherds, of which Mr W. F. Grimes of the Department of Archaeology, National Museum of Wales, states: "The general characters are definite enough to give a date in the Iron Age; comparison with ware from contemporary or subcontemporary sites in South Wales suggests that the present specimens are more likely to belong to the pre-Roman (before about A.D. 50) than to the post-Conquest part of that period. In any case a date not later than the first century A.D. seems certain."

In view of the essentially military character of the Roman occupation of South Wales in its early stages at least the possibility that the trees concerned might have been cultivated by the Romans in Glamorgan may be dismissed, and the conclusion that beech was growing there spontaneously in or before the first century A.D. seems to be inevitable.

A NOTE ON THE OCCURRENCE OF INTERNAL TELEUTOSORI OF *PUCCINIA UMBILICI*

By K. WILSON
Department of Botany, University College, London

Some material of *Cotyledon Umbilicus*, heavily infected by *Puccinia Umbilici*, was found to contain a number of internal teleutosori in the leaf laminae and petioles. These were commonly, though not invariably, formed close to the vascular strands.

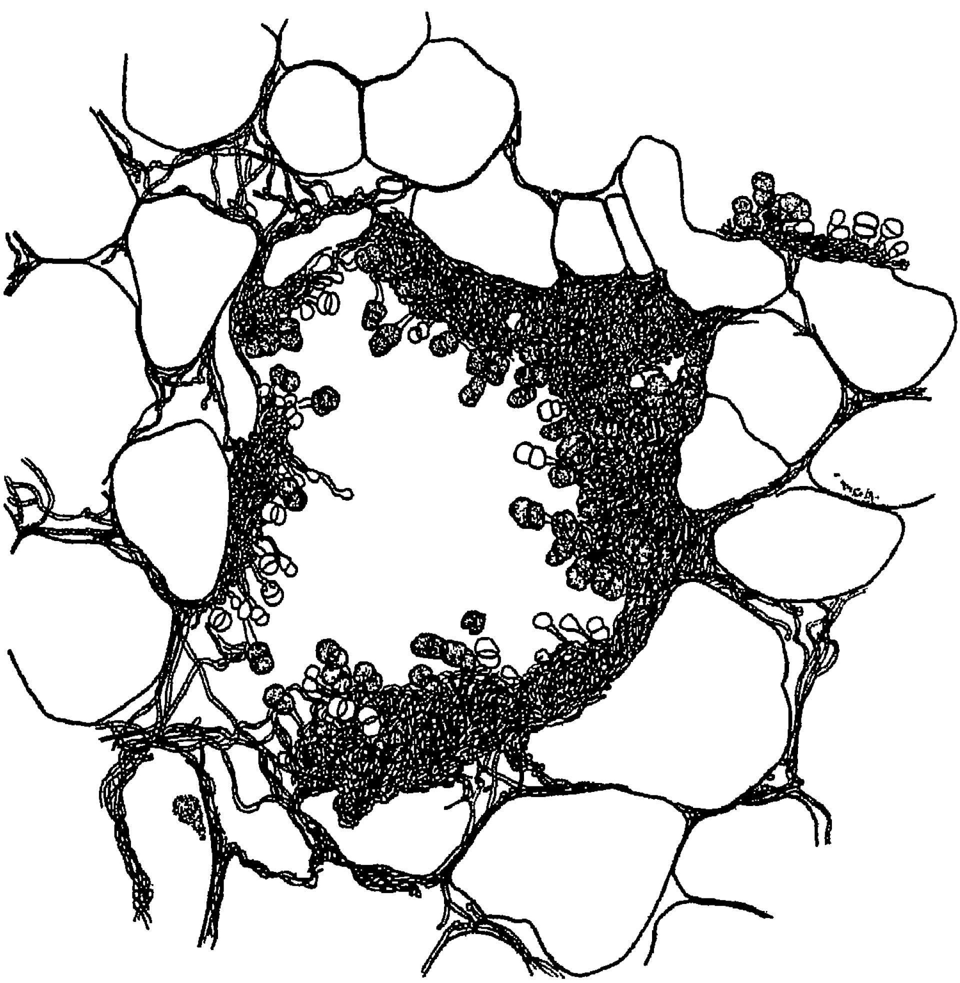

Fig. 1. *Cotyledon Umbilicus*. Transverse section of petiole, showing an internal teleutosorus of *Puccinia Umbilici*. ×125.

The internal sorus develops by an aggregation of fungal hyphae in the intercellular space system of the host to form a pseudoparenchymatous mass, within which teleutospores begin to appear. The sorus so initiated enlarges to a diameter of 300–400 μ, displacing and

disrupting the surrounding host cells as it does so. In this condition it consists of a hollow sphere of closely woven hyphae enclosing a mass of spores. Most of these are free of the mycelium, whilst others are being formed at the periphery. There is no means whereby they can be set free except by decay of the host.

The figure shows such an internal teleutosorus as seen in a transverse section of the petiole of *Cotyledon*. The mature spores which filled the soral cavity in life have fallen out.

Internal sori of rust fungi have been reported previously by other observers; instances are known of the internal formation of teleuto-, uredo- and aecidio-sori as well as of pycnidia. Space does not permit of full citation; references and summaries are given by J. C. Arthur (*Plant Rusts*, 1929, New York: John Wiley and Sons) and by R. H. Colley ("The discovery of internal telia produced by a species of *Cronartium*", *J. agric. Res.* 1917, **8**, 329).

No cause has been assigned to the formation of internal sori. They are commonly associated in position with the vascular tissues of the host, and seem to be formed particularly when the infection and the production of normal erumpent sori are most intense. Presumably under these conditions the fungus is enjoying an optimum environment, and this, acting internally in regions where food substances are most easily available, i.e. near the vascular bundles, results in the formation of internal sori. Especially would this be so in a tissue offering little mechanical resistance to disruption. The centric form of the internal sorus may be ascribed to its comparatively symmetrical surroundings; the asymmetry of environment of the normal sorus initiated near the surface of the host, which leads to its orientation in relation to that surface, is absent.

I am indebted to Mr W. B. Grove, to whom a preparation was sent, and at whose suggestion this note was written.

REVIEWS

Flora des Südens. By C. SCHRÖTER. 5 × 8 in. Pp. viii + 151, with 60 text-figures, 32 coloured plates and 40 black and white plates. Zürich: Rascher. 1936.

This attractive little book gives illustrations of 252 plants to be found in the Southern Alpine valleys of Ticino and Bunden and the neighbourhood of the Italian lakes. 170 of these are natives and the remainder introduced plants of the many beautiful parks and gardens of the district. The book is modelled on Prof. Schroter's well-known *Taschenflora für den Alpenwanderer*, and is intended as a pocket-book for the tourist who is not an experienced botanist, and who is sufficiently interested in plants to wish to know rather more about them than their names. Introductory sections deal with the climate and soil of the region and with the history of its flora. Another gives a list of species likely to be encountered on various botanical rambles, with indications of the status of each, whether native or introduced, endemic or widely distributed, whether submediterranean or alpine, western or eastern, and so forth. The major part is a set of descriptions of the plants illustrated in the many plates These are arranged, not in any taxonomic sequence, but in groups occurring in similar habitats, flowering at the same time, or easily seen during a single excursion. The descriptions include notes on the local and wider distributions, mode of pollination and any other details of general biological interest. Much that is not generally known to the English botanist can be learnt from a casual reading of these notes.

There are no keys to the species, which must presumably be identified by comparison with the plates, help being given by the arrangement into habitat or seasonal groups.

A. R. CLAPHAM.

Methods in Plant Physiology. A Laboratory Manual and Research Handbook. By W. G. LOOMIS and C. A. SHULL. 9 × 6 in. Pp. xviii + 472, with 94 text-figures. New York and London: McGraw Hill. 1937. Price 25*s*.

This is rather a mixed collection of experiments varying from those suitable for school use to "such as will be made by advanced laboratory students or by beginning research workers". The whole field of plant physiology and biochemistry is covered and there is a chapter on the elements of statistics as applied to experimental biology. This consists essentially of a series of examples illustrating the commonly used methods, no exercises are suggested. An appendix supplies some very useful tables of physical constants, composition of buffer mixtures, etc.

The two principal sections of the book, called Laboratory Exercises and General Methods respectively, receive rather different treatments. The first includes all the elementary work and the physiological experiments. The second is a selection of analytical and physico-chemical methods. The first varies from the elementary to the advanced, the second is consistently advanced.

The laboratory methods are, generally speaking, conventional, or only slightly modified from well-known conventions, and should be capable of giving satisfactory results under any reasonable teaching conditions. Experiment 5 and Fig. 9 (exp. 29) are conspicuous exceptions to the general standard.

Exp. 5 includes a "soda-lime drying tower" and invites students of "intermediate" standing to burn 1–2 g. of cotton quantitatively. The difficulties of even moderately accurate organic combustions seem to be quite overlooked Fig 9 shows a porometer "sealed with vaseline to the leaf surface" and making provision for several inches head of mercury.

In the second part, it has not been found possible to give alternative methods of quantitative analysis, which would doubtless have swollen the book most undesirably. It cannot be hoped to please everybody with a single choice, however skilfully the selection is done, so criticism on this score seems condemned beforehand to carping. In only one case does it seem worth risking the accusation It seems unlikely that many plant chemists will want to use the expensive and tiresome chloroplatinate method of estimating potassium; the cobaltinitrite method, in its numerous more convenient and reasonably accurate forms, pretty well holds the field.

W. O. James

The Plant World. By C. Stuart Gager. 9 × 6 in. Pp. v + 136, with 78 text-figures. New York: The University Society; London: Chapman and Hall. Undated. Price 4*s*. 6*d*.

Dr Gager has contributed this volume to a series, subtitled "Highlights of Modern Knowledge", the object of which is "to select and to stress just those points which are interesting and important to know". To present the essence —or perhaps one should say the sugar coating—of all Botany to the layman is a difficult undertaking As is usual in books of this class, some of the statements about a plant's physiology are decidedly queer. Throughout the book the attitude is, naturally, teleological. The following is a fair sample of the contents:

"Not many years ago 'milk emptins' or 'salt rising' bread was often made by the wives of American farmers. An uncovered mixture of fresh milk and cornmeal was set aside overnight in a warm place 'Wild' bacteria got into this from the air or the surfaces of the utensils used, and thus the mixture became a liquid leaven. Mixed with the dough it would cause the dough to 'rise'. No commercial yeast was put in The 'Children of Israel' were not so fortunate when they fled from Egypt Leaving in haste, they were either careless or forgetful about taking with them the 'leaven' or dough saved from previous bread makings. As a result they had only 'unleavened' bread for a time...."

The book is attractively got up.

W. O. James.

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THE ACTION OF AMYLASES IN RELATION TO THE STRUCTURE OF STARCH AND ITS METABOLISM IN THE PLANT. PARTS IV–VII

BY CHARLES S. HANES
From the Low Temperature Research Station, Cambridge

(With 5 figures in the text)

PART IV. STARCH DEGRADATION BY THE COMPONENT AMYLASES OF MALT

THE ACTION OF THE LIQUEFYING AMYLASE (AMYLOPHOSPHATASE)

THE separation of this component from ungerminated barley was reported by Waldschmidt-Leitz & Mayer (1935); observations on its action in a state of isolation are so far restricted to this investigation.

The substrate was paste from potato starch, vigorously stirred in the hot condition (cf. p. 131, this volume). The starch contained 1·2 mg. phosphorus per g. (expressed as P_2O_5) which was not removed by electrodialysis.

The enzyme was shown to cause a considerable fall in the viscosity of starch paste, accompanied by a liberation of bound phosphorus and a slight increase in reducing power. Even by prolonged action, this enzyme appears to be incapable of inducing the profound degradative changes which are normally associated with the action of amylases.

In a typical experiment (Tabelle 9, *loc. cit.*) the outflow time (viscosimeter) fell from about 50 to 30 sec. in the course of 2 hours' action of the enzyme on 2·4 per cent starch paste. (Neither the final value attainable, nor the outflow time for water, is reported.) The reducing power at this time was equivalent to only about 1 per cent of the theoretical maltose, but other experiments indicate that the value rises to 5–6 per cent after prolonged action (e.g. 24 hours, 40°).

With regard to the liberation of phosphorus, there appears a discrepancy between the tabulated analytical figures, and statements made in the text. In Tabelle 9, for example, the figures given for 2 and 24 hours correspond to only 6 and 10 per cent, respectively, of the total phosphorus.[1] It is stated specifically, however, that this latter value corresponds to the total phosphorus content. It appears likely therefore that a consistent tenfold error has been made in presenting the figures.

After prolonged action of the enzyme, products corresponding to 83 per cent of the original starch were recovered by precipitation in 60 per cent alcohol. This material had approximately the same reducing power as the unfractionated products (i.e. 5–6 per cent R_{maltose}), from which it appears that fragments of low molecular weight are not formed to an appreciable extent.

The properties of the isolated product, which is stated to contain no phosphorus, are of considerable interest. It dissolves in warm water to give opalescent solutions. It exhibits a blue iodine coloration which, from the colorimetric observations reported by Samec, Waldschmidt-Leitz & Mayer (1936), appears to be only slightly less intense than that of undegraded starch. The reducing value (by hypoiodite) would indicate a mean chain length of 30–40 units, assuming full reactivity of terminal aldehydic groups. The rotation, $[\alpha]_D$, was 191·5°. This is lower than the value for soluble starch (about 200°) but comes within the range reported for certain starch fractions; for example, Hirst *et al.* reported 190° for amylose separated by the freezing-out method (cf. p. 124).

These properties indicate that the material is still complex and definitely starch-like in nature. It appears accordingly that the enzyme induces with some degree of selectivity the rupture of the linkages which are responsible for the highly colloidal condition of starch paste.

It is tempting to suggest that the final product may represent starch in the form of its individual macromolecules, corresponding to the chains of about 30 glucose units revealed by the purely chemical methods. The reducing power of the disaggregated product is particularly suggestive in this connexion. If this interpretation receives further experimental confirmation, it will be of great interest in suggesting that, with the disruption of molecular aggregations, the

[1] Free phosphorus values of 0·089 and 0·141 mg. per 50 ml. digest are reported at 2 and 24 hours, respectively. 50 ml. contained 1·22 g. starch or 1·4 mg. phosphorus (as P_2O_5).

terminal aldehydic groups of the macromolecules gain their full reducing power.

In applying the term amylophosphatase to this enzyme, Waldschmidt-Leitz & Mayer have emphasized its capacity to liberate esterified phosphorus. In accordance with the view of Samec (cf. p. 132) the small amount of combined phosphorus is considered to be responsible for the polymerized and highly viscous state of potato starch paste, and its hydrolytic removal is held to result in the disaggregation of the highly colloidal structure. The cleavage of phosphorus is accordingly regarded as the essential feature in the liquefaction process.

Two important pieces of evidence are advanced in support of this view. In the first place, alterations in hydrogen-ion concentration (between *p*H 4·4 and 6·2) were shown to have similar effects upon the rates of phosphorus liberation and viscosity change, the optimum being in the vicinity of *p*H 5·6 in each case. In the second place, the preparation was found to hydrolyse glycerophosphate; a preparation of kidney phosphatase, moreover, induced an apparently similar transformation of starch paste. Since the barley preparation hydrolysed glycerophosphate only very slowly, as compared with the kidney preparation (in concentrations which induced similar rates of phosphorus liberation from starch paste), the barley enzyme is considered to be a phosphatase with a high degree of specificity for the phosphoric acid combined in starch.

The reported parallelism between the decrease in viscosity and the liberation of phosphorus clearly favours the view that the esterified phosphorus present in starch plays some essential part in determining its colloidal properties. It is to be noted, however, that even in potato starch, which is relatively rich in phosphorus, phosphoric ester linkages must occur in very small numbers. The content of 0·12 per cent reported by Waldschmidt-Leitz & Mayer indicates, for example, the presence of only 1 atom of phosphorus to about 450 glucose units. On the basis of a chain length of 30 units for starch, this would correspond to 1 phosphoric acid residue per 15 macromolecules. The phosphate might exist in the di- or triester form, as has been suggested by Meyer (1929); it is thus conceivable that 2 or even 3 molecular chains might be linked together through phosphoric ester linkages. There are, however, obvious difficulties in the way of visualizing an association of 15 macromolecules with a single phosphate group, unless it be assumed that the latter serves in some way as a "nucleus of association" of the molecular chains.

It is evident that the information at present available does not establish conclusively that the action of the liquefying amylase on starch paste results exclusively in the cleavage of phosphoric ester linkages, nor that the alteration in properties of starch represents solely the results, direct or indirect, of the hydrolysis of this particular bond. An investigation of the action of the enzyme on starch varieties and fractions (and also glycogen) which contain little or no bound phosphorus, seems to offer a means of shedding further light on the mechanism of the degradation process, and, in particular, of establishing whether or not phosphorus liberation constitutes the pivotal feature of the action of this enzyme.

THE ACTION OF THE SACCHAROGENIC (β) AMYLASE

This enzyme forms the only established example of Kuhn's β-type amylase (in the sense that it liberates products exhibiting β- or dextro-mutarotation—cf. p. 218); for brevity, it will be referred to as β-amylase. The saccharogenic amylases present in wheat and other cereals appear so far indistinguishable from the barley enzyme. Certain of the observations to be discussed were made with preparations from ungerminated wheat.

The distinguishing features of starch degradation by β-amylase are that maltose constitutes almost the sole product of low molecular weight which is formed, and that the hydrolysis comes to an end when approximately 60 per cent of the starch substance has been converted to maltose. After the completed action of β-amylase a blue-violet or violet iodine coloration persists, due to the presence of a residual dextrin (erythrogranulose or α-amylodextrin) which is resistant to the enzyme.

The progress of the starch degradation

In Fig. 6 are shown examples of progress curves illustrating the increase in reducing power (expressed as percentages of the theoretical maltose) during prolonged action of β-amylase on soluble potato starch. The determinations were made by different methods, including copper reagents and hypoiodite. In these different experiments the hydrogen-ion concentration was maintained at or near the optimal value for β-amylase, *p*H 4·7–4·8.

The reaction proceeds relatively rapidly until the reducing power reaches a value corresponding to 50–55 per cent of the theoretical maltose; thereafter greater resistance is evident and the reducing

value rises only slowly to its final level. The differences in the final limits of hydrolysis in the experiments shown in Fig. 6 illustrate the general range of values, from about 60 to 67 per cent conversion, which have been reported by other authors, e.g. Baker (1902), Ling & Nanji (1923), Syniewski (1925), Polak & Tychowski (1929), Hopkins *et al.* (1933).

Part of this variability may be related to differences in the type of substrate preparation used (cf. Fig. 6 A, curves 3 and 4), but some variability inherent in the enzyme preparations may also be suspected. Thus, a number of investigators have reported a relatively sharp cessation of hydrolysis at 60–61 per cent conversion, whereas in other cases it is clear that the reducing power rises very slowly beyond this

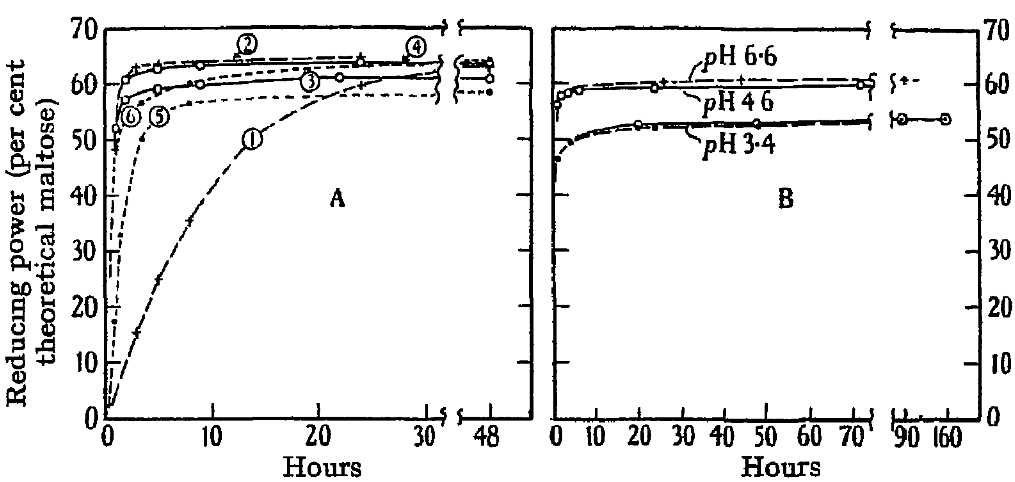


Fig. 6. Progress of starch hydrolysis by β-amylase. A, curves 1 and 2, from Klinkenberg (1932*b*); Lintner starch 1 per cent, enzyme concentration (rel.) 1 and 5. Curves 3 and 4 from Hanes (1935) with Small and Lintner starches (1 per cent), enzyme constant. Curves 5 and 6 from Freeman & Hopkins (1936*b*); Lintner starch 2 per cent. B, from Blom *et al.* (1936); effect of acidity on limit of hydrolysis.

point until values corresponding to 64–67 per cent maltose are ultimately attained.[1] Bearing on this question is the interesting fact recently reported by Blom *et al.* (1936) that in an acid reaction—*p*H 3·4—the hydrolysis ceases sharply at the 53 per cent conversion stage; the same preparations acting at *p*H 4·6 (and 6·8) induced hydrolysis to the normal limit of 60–61 per cent (Fig. 6B).

This observation suggests the presence of some factor in barley extract which normally facilitates the resistant stages of hydrolysis

[1] Unpublished observations of the writer indicate, however, that an enzyme preparation which, when acting on 0·5–1·0 per cent soluble starch, exhibits a sharp limit of hydrolysis at 59–60 per cent, gives higher values (up to 66 per cent) when allowed to act on very dilute substrate, e.g. 0·2 per cent.

beyond 53 per cent conversion, but which does not operate at *p*H 3·4. The enzymes used by Blom *et al.* had been prepared by subjecting aqueous extracts of ungerminated barley to a prolonged acid treatment (several days at *p*H 3·6 and 0°), in order to avoid possible contamination by the dextrinogenic component (cf. p. 137). On this and other grounds it is unlikely that this enzyme constitutes the contributory agency. It seems possible, on the other hand, that the presence of the liquefying enzyme (amylophosphatase) may be found to account for this effect (cf. p. 196). No observations have so far been reported on preparations of β-amylase which are known to be free from contamination by this component.

Finally it may be mentioned that the limit of hydrolysis is not appreciably influenced by wide variations in the enzyme concentration (Klinkenberg, 1931; Hanes, 1935). Moreover, solubilized starches from sources other than potato, e.g. arrowroot, wheat, barley, and rice (Ling & Nanji, 1925); wheat, buckwheat, and arrowroot (Klinkenberg, 1932); maize, apple (Hanes, 1935, 1936), have been found to be degraded to about the same extent as potato starch.

The degradation products

Throughout the hydrolysis, residual starch-like dextrins are present which are readily precipitated by 50–60 per cent alcohol. The ultimate member of this series of products, which remains at the cessation of hydrolysis, is erythrogranulose (Wijsman, 1889) or α-amylodextrin (Baker, 1902). The formation of these "amyloid" dextrins appears to be peculiar to the saccharogenic (β) amylase. These products exhibit iodine coloration ranging from blue to violet, and retain, to a marked degree, the capacity to form molecular aggregates of high dimensions. They are practically non-reducing.

The reducing products of the degradation remain in the alcoholic filtrate after the precipitation of the amyloid dextrins. At the completion of the action of the enzyme, this material was examined by Baker (1902) who found that it agreed closely in both reducing power and optical rotation with pure maltose, and this sugar was isolated in high yield in the crystalline form.[1] These observations have been subsequently confirmed by a number of authors (e.g. Syniewski, 1925; Hanes, 1935; Freeman & Hopkins, 1936 *a*; Blom *et al.* 1936). Owing to the freedom of the product from contamination by

[1] After prolonged action the presence of small amounts of glucose was detected. Evidence was given that this arises from the action of the enzyme on the residual dextrin (α-amylodextrin) rather than from the hydrolysis of maltose.

low-grade reducing dextrins the use of β-amylase has been recommended for the preparation of pure maltose (Baker & Day, 1911; Harding, 1923).

It was reported by Hanes (1935) not only that the properties of the alcohol-soluble material correspond to maltose, but that the yield of precipitated dextrin agreed closely with that calculated on the assumption that the reducing material was exclusively maltose. Confirmatory evidence was also obtained by applying an analytical method based on the use of differential strains of yeasts. The results were in harmony with the view that the reducing material was exclusively maltose, except after prolonged action, when the appearance of traces of glucose was indicated.

Freeman & Hopkins (1936*a*) examined the reducing material present at early stages of the degradation. In two digests, arrested at 14·8 and 24·3 per cent conversion respectively, the alcohol-soluble material was examined. That this consisted exclusively of maltose in each case was attested by the fact that the reducing power/rotation ratio agreed with that of maltose, and that the rates of fermentation by brewers' yeast, and the total carbon dioxide evolution, were not significantly different from the values found for pure maltose.

There is thus good evidence that throughout the whole course of the reaction, maltose constitutes the only low molecular degradation product which is formed in significant amounts. This characteristic, as will be seen later, clearly differentiates the saccharogenic (β) component from other amylases.

Erythrogranulose or α-amylodextrin

The peculiarly starch-like nature of the material which remains at each stage of the degradation has already been mentioned. Most investigators have confined their attention to the ultimate number of this series which remains on the attainment of the hydrolysis limit. In referring to this product the term α-amylodextrin will be adopted, since it serves to emphasize its general starch-like properties.

The considerable theoretical interest attached to α-amylodextrin is related to the problem as to why hydrolysis ceases when this stage is reached. Before considering this question, however, attention may be drawn to a certain variability in the properties of the dextrin as reported by different authors, whose observations are summarized in Table III.

TABLE III. *Properties of residual dextrin (α-amylodextrin or erythrogranulose) after the action of β-amylase*

	Described by	Specific rotation	Reducing power (maltose = 100)	Phosphorus content
1	Wijsman (1889)	—	*ca* 1 (copper)	—
2	Baker (1902)	+190–195°	0·5–2 (copper)	—
3*a*	Ling & Nanji (1923)	(221°)	Negligible (copper)	High
3*b*		193°	,,	None
4	Klinkenberg (1932)	194°	,,	—
5	Freeman & Hopkins (1936*b*)	188°	*ca* 1 (copper)	—
6	Haworth *et al.* (1935)	200°	*ca* 3 (hypoiodite)	High
7	Samec (1936)	193°	*ca.* 16 (hypoiodite)	—

According to Ling & Nanji (1923) the optical rotation of the product is closely related to certain of its physical properties. These authors emphasized, moreover, that the preliminary treatment of the β-amylase preparation markedly influences the properties of the residual body left after the completed action on starch (cf. Nos. 3 *a*, *b*). It is stated that, by the use of an enzyme which has been precipitated and thoroughly dehydrated in strong alcohol, a highly polymerized form of α-amylodextrin is obtained which exhibits high rotation and which retains practically the whole of the phosphorus which was present in the original starch. On the other hand, the action of untreated barley extract, or of a freshly precipitated preparation (which has received only short treatment with alcohol), results in a disaggregated form of α-amylodextrin which exhibits lower rotation ($[\alpha]_D$, +193°) and is entirely devoid of combined phosphorus. This is considered by Ling & Nanji to represent a depolymerized and dephosphorylated derivative of the first type of product.

A possible interpretation of these hitherto inexplicable observations can now be suggested on the basis of the presence or absence of the liquefying component (amylophosphatase) in the β-amylase preparation. According to the observations of Waldschmidt-Leitz & Mayer (cf. p. 189), this enzyme appears to liquefy starch, to liberate gradually its bound phosphorus, and to convert starch into a form which has a small but appreciable reducing power, and lowered rotation.

These effects of amylophosphatase action on the properties of starch itself offer a plausible basis for interpreting the origin of different forms of α-amylodextrin. Thus, the effect of prolonged alcoholic treatment of the enzyme might be assumed to lie in a destruction of amylophosphatase, and the resultant β-amylase preparation would be expected to yield an aggregated, high-rotating type of α-amylodextrin with high phosphorus content and no appreciable reducing power.[1] On the other hand, the presence of the liquefying

[1] The properties of the specimen described by Haworth *et al.* (No. 6, Table III) correspond, according to this view, with α-amylodextrin formed in the absence of active amylophosphatase (i.e. high rotation and phosphorus

component, acting in conjunction with β-amylase, might reasonably be expected to yield a disaggregated, phosphorus-free α-amylodextrin, and the possibility suggests itself that the terminal aldehydic group of such a product might exhibit full reducing power toward hypoiodite (cf. p. 191). This hypothesis, admittedly speculative, might thus account for all the observed variations in the properties of α-amylodextrin; in particular it might explain the otherwise anomalous reducing value by hypoiodite, which has been reported for specimen No. 7 (Table III) by Samec. This value, 16 per cent R_{maltose}, corresponds closely with a chain length of 12 glucose units, assuming stoichiometric reduction by a terminal aldose group, and accordingly it is in agreement with the newer chain-length determinations for this product by end-group assay.[1]

This hypothetical conception of the interplay between the liquefying and saccharogenic components of extracts of ungerminated barley would appear to merit experimental exploration.

Further degradation of α-amylodextrin

The limit of hydrolysis of starch at the stage of about 60 per cent conversion has assumed a position of importance in relation to views of starch constitution (cf. pp. 221 and 228). The cessation of the degradation process is due neither to inactivation of the enzyme, nor to its inhibition by reaction products, as is shown readily by the addition of more starch at this stage. Moreover, isolated α-amylodextrin exhibits high resistance to the action of the enzyme.

The fact must be emphasized, however, that α-amylodextrin, after precipitation and re-suspension in hot water, has never been reported to be *completely* resistant. Some further degradation by β-amylase, however small, appears always to result. The extent of this further breakdown depends, moreover, upon the treatment of the dextrin. Thus, unpublished observations by the writer have shown that mild autoclaving of α-amylodextrin in neutral solution results in a marked decrease in its resistance to the enzyme. After 30 min. at 120°, for example, the dextrin is degraded further by moderate concentrations of β-amylase until a new "resting stage" is reached. The increase in reducing power in different experiments corresponded to 16–26 per cent of the theoretical maltose.[2] In all such cases the iodine colour remained red-violet.

content, negligible reducing power, and highly polymerized state). Although a freshly precipitated enzyme preparation was used, it is perhaps significant that this was subjected to a relatively drastic pre-treatment, being heated to 55° for 15 min.

[1] According to this view, the slow increase in reducing power from 60 to 66 per cent apparent conversion, which is sometimes observed in the action of β-amylase on starch, might be due to the transformation of the aldehydic end unit of α-amylodextrin into a reducing form, and not to the liberation of an additional maltose molecule (cf. p. 228).

[2] The nature of the reducing products has not been investigated.

These observations are of interest in relation to the experiments of Hopkins *et al.* (1933), who followed the hydrolysis of soluble starch by the repeated action of relatively enormous concentrations of β-amylase. The resistant material after each digestion was isolated and, in preparation for the succeeding treatment with enzyme, was re-dispersed by autoclaving.[1] Each repetition of the treatment induced further degradation; reducing products were liberated (from the osazone test, apparently maltose and glucose) and the amount of the resistant dextrin fraction diminished. Finally, after six treatments, this corresponded in yield to only 3 per cent of the original starch.

The successive dextrin residues, which became progressively more resistant to degradation, retained the iodine colouring property; this changed finally from violet to pale brown. These products appear to constitute additional members of the series of amyloid dextrins which in the normal hydrolysis of starch (without autoclaving) terminates when the α-amylodextrin stage is reached.

It is clear, therefore, that under these conditions (successive additions of fresh enzyme, interspersed with autoclaving) a far-reaching degradation of starch, of the order of at least 90 per cent conversion, is attainable. The observations indicate that, whatever may be the precise nature of the obstruction to the action of β-amylase which determines the cessation of hydrolysis at the α-amylodextrin stage, this impedance is to some extent removed by relatively mild autoclaving treatment.

Brief mention should also be made of another fact which bears on this question. α-Amylodextrin, although resistant to β-amylase, is readily degraded by the dextrinogenic (α) malt amylase; this results in the destruction of the iodine colouring property, and the liberation of reducing products. Klinkenberg (1932) reported that certain of the mixed degradation products in this case were further degraded by β-amylase. Hanes (1935), in confirming this observation, reported that this effect is to be observed almost from the beginning of degradation by the dextrinogenic amylase.

It is clear, therefore, that by the action of dextrinogenic amylase, portions of the molecular structure of α-amylodextrin are liberated which are readily attacked by the saccharogenic (β) amylase.

Degradation of amyloamylose

It has been reported frequently that amylose and amylopectin, separated in various ways, are hydrolysed at markedly different rates by amylases. A higher rate of hydrolysis of the more soluble amylose

[1] I am indebted to Prof. Hopkins for permitting me to mention the fact (which was communicated privately) that autoclaving was adopted as a means of re-dispersing these products. This was not specifically mentioned in the original paper.

is perhaps to be expected purely on the basis of its physical state; the fact that soluble starch is degraded more rapidly than starch paste would appear, at least superficially, to offer a parallel example.

From time to time, however, the view has been advanced that amylose and amylopectin are *qualitatively* different as substrates for amylase action. Thus, Ling & Nanji (1925) state that amylose (separated by the "freezing-out" method, cf. p. 123) is completely transformed into maltose by either malt amylase (i.e. α-+β-malt amylase) or by barley amylase[1] (i.e. β-amylase).

Having concluded that β-amylase transforms the amylose fraction completely into maltose, the further assumption was made by Ling & Nanji, with no supporting evidence, that the amylopectin fraction is not hydrolysed at all by this enzyme. A new definition of "amylopectin" was accordingly created, namely any starch product which is resistant to hydrolysis by β-malt-amylase. In this way was initiated the view, subsequently developed by van Klinkenberg (cf. p. 221), that the cessation of starch degradation by β-amylase at the 60–65 per cent transformation stage is due to the selective hydrolysis of a pre-existing fraction of the starch substance.

While the important contention of Ling & Nanji, that the amylose fraction of starch is completely hydrolysed by β-amylase, has not been confirmed in some subsequent investigations,[2] there is now considerable evidence to show that at least one particular form of amylose, namely the amyloamylose of Samec and Mayer (cf. p. 123), is degraded almost to completion by this enzyme.[3] This observation, first reported by Samec & Waldschmidt-Leitz (1931), has been well substantiated (Freeman & Hopkins, 1936*b*; Samec, 1936; and unpublished observations of the writer, 1936).

Amyloamylose is hydrolysed much less rapidly than soluble starch. After 60–70 per cent has been transformed by the action of β-amylase, the residual material shows increasing resistance to hydrolysis, but reduction values corresponding to 95–98 per cent of the theoretical maltose are finally attained.[4] A blue iodine coloration is reported to

[1] This was supported by a single experiment, briefly described (*loc. cit.* p. 633).

[2] Hirst *et al.* (1932) reported that amylose and amylopectin, prepared by the Ling & Nanji method, are both hydrolysed to the same extent by β-amylase and that they were indistinguishable from starch itself in this respect. This is also true of amylose separated from a cold water suspension of milled starch (p. 130) according to unpublished observations of the writer. A hydrolysis limit of 58 per cent was observed.

[3] And also by pancreatic amylase (Samec, 1936).

[4] Whether or not the products are exclusively maltose has not been

persist up to the stage of 80–90 per cent apparent conversion, and this then changes with further degradation to violet and pink.

In certain respects, the degradation of amyloamylose under the action of β-amylase resembles the far-reaching conversion of soluble starch which was attained by periodic resort to autoclaving, a resemblance which possibly merits emphasis on account of the fact that autoclaving forms a preliminary step in the preparation of amyloamylose.

In assessing the significance of this development, the general features of the procedure for separating amyloamylose must be considered. Paste from potato starch is first autoclaved (e.g. 30 min. at 120°), and then subjected to electrodialysis. A gelatinous fraction gradually migrates toward the positive membrane, situated at the bottom of the cell. At the completion of this process, the amyloamylose fraction (representing about one-fifth of the total starch) remains in the supernatant solution and it is this fraction which is hydrolysed almost to completion by β-amylase. The major fraction which has settled, however, is not considered to represent erythroamylose in a pure form. To obtain this component, it is necessary to subject the "crude" erythroamylose to repeated electrophoretic fractionation, the precipitated material being re-dispersed at each stage by autoclaving. The repetition of this process results in diminishing amounts of the product, with an accompanying removal (or generation) of amyloamylose. Whether or not these subsequent crops of amyloamylose are also completely degraded by β-amylase has not been reported. Ultimately, after five or six treatments, erythroamylose is obtained, recognizable by a red-violet coloration with low iodine concentration, and red with excess iodine. The two starch fractions prepared in this way are devoid of combined phosphorus and in each case the product exists in a highly aggregated state. This is clear from measurements of osmotic pressure (cf. Samec, 1927) and from their behaviour in solution.

Although the method of electrodialysis has resulted in the separation of a starch fraction which appears to be almost completely hydrolysed by β-amylase, it is to be noted that it has afforded no evidence for the existence of a fraction which is abnormally resistant to this enzyme. Freeman & Hopkins (1936*b*), for example, who obtained erythroamylose in 60 per cent yield, reported its hydrolysis to the extent of 58 per cent conversion. This is in close agreement with the value of 56 per cent reported by Samec (1936). These values, which are slightly lower than the usual limit of hydrolysis of soluble starch, are probably not appreciably lower than the values which would be observed for autoclaved starch paste under comparable conditions. This point is of considerable importance, since it disposes of the view that the limit of hydrolysis of β-amylase acting on soluble starch is due to a selective hydrolysis of the amyloamylose fraction (cf. p. 222).

The difference in the behaviour of amyloamylose and erythroamylose as substrates for amylase action is clearly a question of great interest. No finality of interpretation is to be expected until these products have been examined by the modern chemical methods, and in particular by end-group assay. Such results might be expected to indicate whether these differences are related to molecular constitution (chain length) or whether an explanation is to be sought in the degree or mode of association between similar macromolecules. Finally, it seems important that the role of factors which operate during the separational procedure (especially the repeated autoclaving) should be further analysed in order that the relationship between these fractions and whole starch may be clarified.

Changes in iodine coloration

The term "saccharogenic" was originally intended to signify that this amylase causes the transformation of a considerable portion of the starch substance into maltose without destroying the iodine colouring property. As might be expected, the actual intensity of the iodine colour decreases as the concentration of the amyloid dextrin residue becomes progressively smaller, and there is a noticeable change in hue, from blue to blue-violet or violet, as the liberation of maltose proceeds.

The decrease in colour intensity has been reported by Samec (1936) and Blom *et al.* (1936), who have made observations with Dubosq-type colorimeters (using starch solutions with the same iodine concentration for comparison).

Hanes & Cattle (1937), using a method of approximate spectrophotometry, have investigated the absorption characteristics of the coloured iodine complexes at different stages of the degradation of starch by various amylases. With the saccharogenic (β) amylase, the absorption spectra during the degradation retain a general similarity to that of undegraded starch, in that the position of maximal absorption does not shift. This contrasts sharply with the changes induced by amylases of the dextrinogenic type (cf. p. 208). It was found that the extinction coefficient in the region of maximal absorption decreases approximately (but not exactly) in proportion to the concentration of residual dextrin. Actually, after 30–40 per cent conversion, the residual dextrins exhibit an iodine coloration which is similar but appreciably more intense than that of undegraded starch in the same concentration; accordingly during the early stages

of degradation the percentage decrease in colour is less than the percentage conversion of the substrate into maltose.

The mode of attack by saccharogenic (β) amylase

A consideration of the different characteristics of the action of the saccharogenic (β) amylase leads to a unified and simple conception of the degradation process induced by this enzyme. Thus, a number of lines of evidence point to the conclusion that this amylase induces an end-wise type of degradation, in which successive terminal disaccharide fragments are split off from one end of the molecular chain structure of the substrate.

In considering the evidence leading to this conclusion, considerable weight must be attached to the fact that maltose constitutes almost the sole product of low molecular weight which is formed from the beginning to the end of the normal action of the enzyme on starch. This peculiarity of the reaction (which distinguishes β-amylase from amylases of the dextrinogenic type) would be inexplicable except by assuming an exclusively end-wise mode of attack; on any alternative basis, the production of short chain (reducing) fragments other than maltose would seem inevitable, particularly during the early stages of degradation.

This conception is firmly supported by the characteristics of the amyloid dextrins which, together with maltose, are formed in the course of the degradation. The peculiarity of this series of dextrins is their close similarity in many properties to undegraded starch (p. 194). From this alone, it is clear that they represent large intact residues of the starch molecule, and that they are not products of an extensive fragmentation of the original structure. This view of the manner of formation of the amyloid dextrins is supported more specifically, however, by two separate pieces of evidence.

Ohlsson (1926) investigated the osmotic behaviour of the products when soluble starch was degraded to the extent of 31 per cent conversion into maltose by this amylase. The osmotic pressure was initially high, but after the lapse of sufficient time for the equilibrium distribution of simple sugars through the collodion osmometer sac (48 hours), the osmotic pressure was found to be slightly lower than that found for the undegraded starch.[1] It was accordingly clear that no increase in the number of non-dialysable particles had accompanied

[1] This is to be compared with an eight- to ninefold increase in osmotic pressure as a result of degradation by the dextrinogenic (α) component (cf. p. 210).

the partial transformation of the substrate into maltose. On the basis of this observation, Ohlsson advanced the view that by the action of this enzyme successive maltose molecules are detached from each starch molecule in such a manner as to leave at each stage a single residual molecule of dextrin. This view adumbrates the conception which is now under consideration.

Furthermore, the establishment of a chain length of 12 units for α-amylodextrin constitutes convincing evidence that this product represents an intact residual portion of the original starch molecule structure. Thus, on the basis of a chain length of 30 glucose units for starch, the removal of nine successive disaccharide fragments (which corresponds to a 60 per cent conversion of the substrate into maltose) would leave a residual dextrin molecule containing 12 glucose units.

On the basis of these different lines of evidence it can be concluded with assurance that the saccharogenic (β) amylase acts exclusively by inducing the liberation of successive terminal maltose fragments from the chain molecule of the substrate, and the question arises as to which end of the structure is involved in this process.

The existing information suggests that the degradation by β-amylase must proceed from the non-aldehydic end of the starch chain structure. The fact that the amyloid dextrins (including α-amylodextrin) are normally almost non-reducing supports this view, since it suggests that the liberation of maltose proceeds without disturbing the configurational features which render non-reactive the aldehydic end-group of starch itself. More direct support is obtained from observations of Brown & Millar (1899) on the enzymic degradation of a dextrinic acid. This was prepared by the oxidation of a so-called maltodextrin, which contained about 6 glucose units per molecule (cf. p. 212), with mercuric oxide and baryta. In this way short chain fragments, terminated at the aldehydic end by a modified carboxylated unit, were prepared.[1] This product was found to be readily degraded by normal (i.e. α- + β-) malt amylase, yielding maltose and a dextrinic acid of lower complexity. Since the original dextrin is attacked only slowly by α-malt-amylase it is probable that this degradation was due almost exclusively to the β-component. These observations accordingly constitute strong evidence that the aldehydic end of the chain is not involved in the combination with this amylase.

[1] Under these conditions oxidation proceeds farther than the conversion of the aldehydic C_1 group to a carboxyl group.

Nomenclature

The name β-amylase, which is now widely used, emphasizes the fact that the degradation products exhibit dextro- (or β-) mutarotation. This has been interpreted as arising from the selective hydrolysis of β-linkages assumed to be present in the starch molecule. For reasons which will be discussed later (cf. p. 224) this view can no longer be accepted, and it is now clear that the β-mutarotation, by itself, does not constitute a definition of the specificity of this enzyme.

The older term "saccharogenic" emphasizes more directly the peculiarity of the action of this amylase—that maltose constitutes almost the sole reducing product. This name might be considered open to objection on the ground that dextrins are produced during the degradation. In proposing this name, Ohlsson (1926) pointed out, however, that any fragmentation of the starch molecule must lead to the formation of products which, by definition, are dextrins, and he stressed the fact that, in the case of this particular amylase, the dextrins which are formed represent merely residual portions of the original structure left after the detachment of individual maltose fragments.

Both current systems of nomenclature appear now to be descriptive of the degradation products rather than definitions of the specific constitutional requirements of the enzyme. It seems likely that knowledge of the saccharogenic (β) amylase will be sufficient in the near future to permit the formulation of a more ideal terminology.

THE ACTION OF THE DEXTRINOGENIC (α) AMYLASE OF MALT

The dextrinogenic malt component, in contrast to the saccharogenic (α) amylase, readily induces the destruction of the iodine colouring property of starch. It forms one of the three amylases which have been shown to belong to Kuhn's α-type (cf. p. 218) and for convenience may be referred to as α-malt-amylase.

The liberation of reducing products

Observations on the liberation of reducing groups during the degradation of soluble starch by α-malt-amylase have been reported by a number of authors. The data display minor differences as to detail but are in agreement as to the main features of the reaction progress.

In Fig. 7 A, B, C, D, are shown progress curves from the data of van Klinkenberg (1932), Holmbergh (1933), Hanes (1935), and Freeman & Hopkins (1936*b*). In the first three cases reducing power

was determined by alkaline copper methods (but with different reagents in each case), whereas Freeman & Hopkins used the hypoiodite method. Holmbergh's enzyme was separated from malt extract by four successive adsorptions on rice starch (cf. p. 139); in the other three investigations, preparations of the Ohlsson type (but with 15 min. heating at 70°, cf. p. 137) were used.

For the purpose of comparison, a progress curve reported for a bacterial amylase by Blom *et al.* (1936) has been included in Fig. 7B.

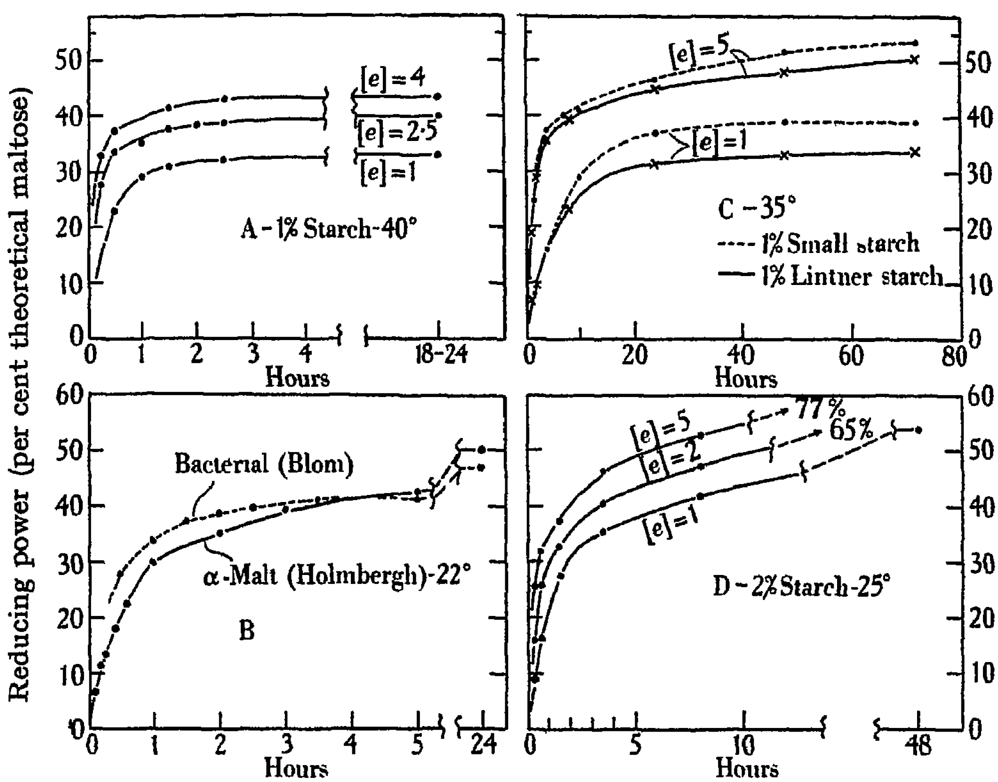


Fig. 7. Progress of starch hydrolysis by α-malt-amylase A, from Klinkenberg (1932*b*); B, from Holmbergh (1933) (with observations by Blom *et al.* (1936) on a bacterial amylase); C, from Hanes (1935); D, from Freeman & Hopkins (1936*b*).

This preparation appears to be closely similar to the dextrinogenic malt component (cf. p. 217).

On the basis of observations of which these are typical, the following conclusions can be drawn:

(1) When sufficient enzyme is present, the progress curve exhibits two distinct stages—an initial phase in which the reducing power rises rapidly to a value equivalent to 30–40 per cent of the theoretical maltose, followed by the second prolonged phase of extremely slow increase in reducing power. The transition between these two phases

appears as an inflexion, varying in its sharpness in the different curves, but discernible in all.

(2) By increasing the enzyme concentration, not only is the initial velocity increased, but the degree of hydrolysis attained after prolonged action is appreciably raised.

The most striking feature of the reaction is the more or less abrupt termination of the initial rapid phase of hydrolysis at a reducing level corresponding to 30–40 per cent of the theoretical maltose value. In a number of cases slope values measured after the transition represent only about one-twentieth of the slopes measured before this point.

An examination of the different progress curves in Fig. 7 reveals considerable variability as regards the general form and position of the transition between the two reaction phases. Of the curves based on copper values, the single example with Holmbergh's enzyme—B—shows an inflexion at an appreciably lower reduction level (about 29 per cent R_{maltose}) than sets A and C in which it occurs within the limits 32–38 per cent R_{maltose}. The data of Freeman & Hopkins—D—which are based on the hypoiodite method, differ considerably in that the inflexions are less clearly defined and occur at appreciably higher levels of reducing power.

During the prolonged reaction periods necessitated by the extreme slowness of the second phase, it is possible that progressive inactivation of enzyme becomes a factor in determining the course of the degradation. This may partly explain the dependence of the limit of hydrolysis upon the amount of enzyme added initially.

The maximal extent of degradation which can be induced by α-malt-amylase is not clearly established. In the majority of reported experiments the reducing power virtually ceases to rise at levels corresponding to 45–60 per cent of the theoretical maltose value, but in certain cases considerably higher values have been observed when very high enzyme concentrations were added. Thus, Klinkenberg reported values corresponding to 75 and 78 per cent conversion in two experiments, and Freeman & Hopkins, 77 and 84 per cent. In view of possible contamination of the preparations by slight traces of active β-amylase, these observations, based on the use of relatively enormous additions of enzyme, are not entirely conclusive. (It is to be noted that the limit of hydrolysis in these cases is approximately the same as that observed when starch is degraded by α- + β-amylases, cf. Blom *et al.* 1936).

A consideration of the available information suggests that with pure α-malt-amylase the termination of the initial rapid phase would

occur at about 28–30 per cent apparent conversion, and that the limit of hydrolysis would not exceed a value of approximately 50 per cent R_{maltose}. That this degree of purification is obtainable by the Holmbergh method, and, for certain malt extracts, by the Ohlsson method, is indicated by unpublished observations of the writer.

Destruction of the iodine colouring property

(1) *Changes in colour.*

The colour developed with iodine undergoes rapid transformation from blue, through violet, to shades of red-brown. The subsequent alterations (through orange, and deep yellow) occur more gradually and the recognition of the actual achroic point is a matter of some difficulty.

Hanes & Cattle (1937) have followed the colour changes quantitatively by means of a spectrophotometric method. Digest samples equivalent to 10 mg. of the original substrate were removed at intervals and mixed with 5 ml. 0·2 per cent iodine. After dilution to 100 ml., extinction coefficients were measured over a range of spectral bands. Under these conditions, it was found that for starch, and a number of dextrins, the extinction coefficients (after deducting the values observed for iodine alone at the corresponding wave-lengths) were proportional to the concentration of starch products. The liberation of reducing groups was followed at the same time, determinations being made by both alkaline copper and hypoiodite.

In Fig. 8A is shown an example of observations made by this method during the degradation of 0·2 per cent soluble starch by α-malt-amylase, and for comparison, observations on a corresponding digest by β-malt-amylase (Fig. 8B).

In the case of α-malt-amylase, the initial stages of degradation are seen to result mainly in decreased extinction of the longer wave-lengths, with little change toward the blue end of the spectrum. This indicates increasing transparency of the solutions to red light. Later, the values fall throughout the whole spectrum, but the fall continues to be more pronounced at the longer wave-lengths. This results in a marked shift in the absorption maximum toward the blue end, until the curves gradually approximate to the general form of that of the iodine alone (without added starch products). A closely similar sequence of changes was observed during degradation of starch by other amylases of the dextrinogenic type (*Aspergillus oryzae*, pancreatic and salivary amylases).

During degradation by the saccharogenic (β) amylase, on the other hand, the extinction values decrease from the beginning over the whole spectral range, the positions of maximal absorption and *general* form of the extinction curve remaining unchanged (Fig. 8B). Thus even at the latest observed stage (at which the reduction value was 59 per cent of the theoretical maltose) the curve shows a general similarity to that of undegraded starch.

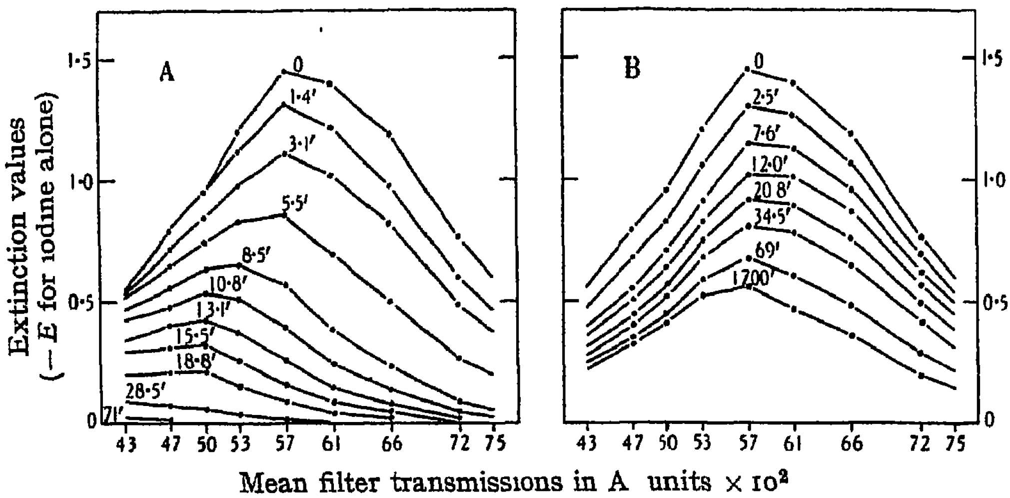


Fig. 8. Alteration in iodine coloration during starch hydrolysis: A, by α-malt-amylase; B, by β-malt-amylase.

(2) *The reducing value at the achroic point.*

The reducing value at the achroic point (*achroic R value*) gives an indication of the number of linkages which have been hydrolysed, at the stage at which the iodine colouring property is completely destroyed. For reasons which have been discussed earlier (p. 134), it is probable that the achroic stage is reached when dextrins containing 7–8 glucose units, or more, are no longer present.

On this basis, the ideal "dextrinogenic" process of starch degradation (that is, the destruction of the iodine colouring property with the liberation of the minimal number of reducing groups) would consist in the transformation of the whole of the substrate into a dextrin of 6–7 units, this, so far as is known, representing the longest fragment which is devoid of iodine coloration. In this hypothetical case, the reducing power of the 6–7-unit dextrin product, assuming full reactivity of the aldehydic end-groups, would correspond to 29·7–34·6 per cent of the theoretical maltose and this would thus represent the lowest attainable *achroic R value*.

The application of this semi-quantitative reasoning to the de-

gradation processes induced by different dextrinogenic amylases is of some interest.

The *achroic R value* has been used in the past as a criterion of the so-called dextrinogenic/saccharogenic ratios of different amylase preparations, low values being taken to indicate a more specialized capacity of an enzyme to destroy the iodine colouring property, and higher values a less specialized "dextrinogenic" capacity. Of the investigated examples of dextrinogenic amylases, α-malt-amylase is characterized by the lowest *achroic R value* (cf. p. 217) and it seems clearly significant that this approximates closely to the lowest attainable *achroic R value* predicted from the considerations above.

Thus, various early workers have reported *achroic R values* corresponding to 28–31 per cent of the theoretical maltose value (determinations by Fehling's solution). A re-examination of this question by Hanes & Cattle (1937), by means of the spectrophotometric method mentioned earlier, has confirmed this value. The iodine colouring property disappears when the reducing power is equivalent to 29–32 per cent R_{maltose}, the value being approximately the same whether determined by copper or hypoiodite reagents.

Other dextrinogenic amylases which were examined gave higher values. Moreover, whereas the *achroic R value* (expressed as a percentage of the theoretical maltose) for α-malt-amylase is found to be practically independent of the substrate concentration, the amylases of *Aspergillus*, pancreas and saliva exhibit increasing *achroic R values* with increasing substrate concentration.

The two points which deserve emphasis are that the achroic stage is reached at or about the end of the initial phase of rapid degradation, and that the *achroic R value* for this enzyme corresponds with that which would be expected to result from an "ideally dextrinogenic" transformation, namely its conversion into a dextrin of 6–7 glucose units.

The nature of the degradation products

There is abundant evidence to show that during the initial rapid phase of degradation by α-malt-amylase the predominating products are dextrins, and that the reducing power during this phase is due mainly to the presence of dextrins of reducing character. This fact requires particular emphasis in view of a tendency on the part of many authors in recent years to assume that maltose is invariably the exclusive or principal reducing product present at all stages of starch breakdown by amylases (cf. p. 223).

The separation and identification of dextrins of the type formed

by this and other dextrinogenic amylases is a matter of great technical difficulty. Precise information concerning these degradation products is essential, however, for any detailed conception of the manner in which the starch molecule is degraded under their action. In recognition of this fact, a number of detailed and painstaking investigations were conducted toward the close of the last century.

Before considering these important early observations, attention will be directed to two more recent studies which establish the general features of the process.

Ohlsson (1926, 1930) investigated the osmotic behaviour of the products during the initial phase of starch breakdown by α-malt-amylase. The reaction was arrested when the iodine colour was violet, and the reduction value corresponded to 23 per cent theoretical maltose. The solution was placed in a collodion sac osmometer, and after 2 days (during which time maltose would have diffused evenly) the pressure stood at 407 mm. of water, as compared with 58 mm. for the undegraded substrate. During the following 5 days the pressure fell slowly to 227 mm.; this was interpreted as a slow diffusion of particles considerably more complex than maltose through the membrane. At this time (7 days) the reducing power of the inner liquid was approximately four times that of the outer liquid. An examination of the diffusate (in the outer liquid) showed it to exhibit a substantially lower reducing power than maltose.

Ohlsson's observations established that a great increase in the number of non-dialysing (or slowly dialysing) particles had occurred and that these relatively complex products accounted for the bulk of the reducing power.

Freeman & Hopkins (1936*a*) subjected similar products to fractionation on the basis of their solubility in alcohol. In two digests the action of α-malt-amylase was arrested when the reducing values corresponded to 20·6 and 25·6 per cent respectively of the theoretical maltose, the iodine coloration being red-violet in each case. After evaporation to dryness, the products were exhaustively extracted with boiling 95 per cent alcohol. In this way alcohol-soluble "sugar" fractions and alcohol-insoluble dextrin fractions were obtained.

The major fraction, about 80 per cent in each case, was insoluble in alcohol and consisted of dextrins readily soluble in cold water, and showing no tendency to retrograde when stored in solution. These dextrins, although free from maltose, showed considerable reducing power, the values in the two cases being 20·3 and 23·0 per cent R_{maltose}. It will be noted that these values are approximately the same as the reduction values of the mixed degradation products before fractionation (i.e. 20·6 and 25·6 per cent respectively).

An examination of the alcohol-soluble "sugar" fractions demon-

strated that this material, although it probably contained some maltose, was composed to a considerable extent of fragments more complex than maltose. This was shown by observations on optical rotation, reducing power, and rates of fermentation by brewers' yeast (cf. p. 195).

While these two investigations leave no doubt as to the general character of the degradation process, they provide no clues as to the range of molecular size of the reducing dextrins which clearly form the principal products. Information bearing on this point, however, is deducible from the observations which will now be considered.

Early observations on reducing dextrins

As a result of O'Sullivan's classical investigation (1876) it was known that whereas the principal end-product of starch degradation by malt amylase (i.e. α- + β-malt-amylase) is maltose, intermediary products are formed which exhibit reducing power, but which could not be induced to crystallize, nor to form crystalline phenylosazones. It was soon recognized that by "limiting" the action of malt amylase, either by previously heating the extract to 70–80° or by allowing the action to proceed at this elevated temperature, maltose production is greatly reduced and, under certain conditions, practically the whole of the starch is converted into non-crystallizable reducing dextrins of this type.

It is now clear that in many of these early experiments the action of α-malt-amylase, in isolation, was under investigation; in others appreciable contamination by β-malt-amylase may be suspected.

The still-complex products of the action of "heat-restricted diastase" were found to be further degraded with great rapidity by "normal" malt amylase (cf. p. 203).

It was recognized that the mixed products formed from starch under conditions of "dextrinogenic" degradation comprised a series of compounds of varying chemical complexity. It was found that these mixtures could be fractionated on the basis of the differential solubilities of the components in hot and cold alcohol-water mixtures of varying concentrations. By this method an extensive range of fractions was separated for examination. It became clear that the solubility of these products was closely related to their molecular complexity. Thus the less soluble fractions displayed higher rotational values, high molecular weight as determined cryoscopically (freezing-point in aqueous solution), and low reducing power; amongst these more complex dextrins were the so-called amylodextrins and erythrodextrins exhibiting, respectively, blue (or blue-violet) and red colorations with iodine. With increasing solubility in alcohol, the dextrin

fractions showed lower rotation, increasing reducing power, and greater depression of the freezing-point. Amongst such lower products were the achroodextrins and maltodextrins, and the so-called isomaltose fractions.

In Table IV are set out the properties of two distinctive dextrin fractions which were isolated by different investigators from the products of "heat-restricted" malt amylase. In each case the products are hygroscopic; they form no crystalline osazones, and are fermentable not at all or only very slowly by brewers' yeast. Type A dextrin is soluble in hot 80–85 per cent, but sparingly soluble in hot 90 per cent alcohol. Type B dextrin dissolves in hot 90 per cent but is only slightly soluble in hot 95 per cent alcohol.

TABLE IV. *Dextrin fractions (maltodextrins or achroodextrins) from the products of "heat-restricted" malt amylase*

Described by	Reducing power (Maltose = 100)	Specific rotation	Molecular weight (cryoscopic)
Type A:			
Brown & Morris (1885)	34·5	180°	*ca.* 1000
Ling & Baker (1897)	31·5–33	178–180°	930–950
Brown & Millar (1899)	(42)	181–183°	—
Syniewski (1902)	30	179·5°	1040
Polak & Tychowski (1929)	31	—	—
Type B:			
Ling & Baker (1897)	43	172°	670
Prior & Wiegman (1900)	42·5	171°	640
Syniewski (1902)	43	172°	595

C. Expected properties of short-chain dextrins (see text):

No. of glucose units	3	4	5	6	7	8
Reducing power (maltose = 100)	68	51	41	35	30	26
Specific rotation	+159°	170°	177°	181°	185°	187°
Molecular weight	504	666	828	990	1153	1314

Dextrin type A is of particular interest since this appears to constitute the main product which is formed during the initial phase of starch degradation by α-malt-amylase. Thus Syniewski (1902) reported yields of 80–90 per cent of this material when the reaction was arrested at the achroic point. The reducing power and optical rotation of the isolated dextrin fraction were reported, moreover, to be closely similar to the corresponding properties of the unfractionated products at this stage. There is no doubt that the enzyme was virtually free from β-amylase in Syniewski's experiments since malt extract was pre-heated to 76–78° for 15 min.

In addition to dextrin fractions of type A, other investigators have encountered less complex material including dextrins of type B, and mixtures of shorter chain fragments. It seems likely that the formation of such products in appreciable quantity may occur only during

the secondary slow phase of the degradation or in the presence of β-amylase. Thus, Syniewski observed the formation of a dextrin of type B as an intermediary product during the action of "normal" (i.e. α- + β-) malt amylase on the type A dextrin.

All reported fractionations of the products of "heat-restricted" malt amylase appear to have resulted in the isolation of considerable amounts of the type A dextrin fraction. The close similarity in properties of the products isolated under varying conditions by different investigators suggests, moreover, that this is a well-defined product of at least approximate chemical homogeneity. This view is supported by the results of Brown & Morris (1885), who were unable to separate this product into fractions having different properties (R, and $[\alpha]_D$) either by repeated fractionations in alcohol, or by allowing the dextrin to diffuse slowly through a porous membrane.

It is to be noted that the specimen described by Polak & Tychowski (Type A, Table III) was obtained in 87 per cent yield from the degradation of the amylose fraction of starch.

In Table IV C are set out, for dextrin molecules containing 3–8 glucose units, the predicted values of reducing power (assuming stoichiometric reduction by a terminal aldehydic group), $[\alpha]_D$ (from Freudenberg's equation[1]), and the molecular weight. Each of these properties is clearly open to some objection as a criterion of chain length. Thus reduction values by Fehling's solution would not be expected to exhibit a strictly stoichiometric relationship; the applicability of the optical super-position rule is not established for the free products,[2] and the possibility of contamination of the products by alcohol demands cautious interpretation of observations on the freezing-point depression. It is worthy of mention, however, that the properties of the "hexaose" described by Waldschmidt-Leitz and Reichel (1934) are in close agreement with the predicted values for a 6-unit dextrin (reducing power by hypoiodite 34·4 per cent $R_{maltose}$; $[\alpha]_D$, 182·5°; molecular weight, ebullioscopic, 960, 1010). This dextrin was isolated from the products of the action of pancreatic amylase on erythroamylose and rice starch, cf. p. 217.

By comparing the reported values of R, $[\alpha]_D$, and molecular weight for the various specimens of the two dextrin fractions with the predicted values, it will be seen that the different properties indicate consistently a mean chain of about 6 glucose units for type A, and about 4 glucose units for type B.

These observations accordingly suggest that the A-type dextrin, which appears to be formed invariably during starch breakdown by α-malt-amylase, and which has been isolated in high yield under some

[1] The calculations were based on the following values: for starch, $n=30$, $[\alpha]_D=200°$; for maltose, $[\alpha]_D=138°$.

[2] This presupposes the same equilibrium mixture of α- and β-forms for dextrins of varying chain length.

conditions, is composed of fragments of the starch molecule containing about 6 glucose units. The potential unreliability of the criteria upon which this conception rests, and, despite indications to the contrary, the possibility of some appreciable heterogeneity of the product with regard to chain length, emphasizes the desirability of the examination of this product by the modern methods which have been described earlier (p. 114). There seems little doubt, however, that these early investigations give an approximate idea of the molecular dimensions of these products.

Mode of attack by α-malt-amylase

The process of starch breakdown under the action of this amylase is more complex than that induced by the saccharogenic (β) amylase, and less clearly defined by the existing data. The broad features, however, are firmly established.

Various lines of evidence indicate that during a primary phase of rapid hydrolysis, the bulk of the substrate is converted into still-complex chain fragments of reducing character (i.e. reducing dextrins) with the formation of only small amounts of products of low molecular weight. From the beginning, the iodine coloration alters progressively, and the achroic condition is reached when the reducing power corresponds to 29–32 per cent of the theoretical maltose. This coincides approximately with the termination of the primary rapid phase of hydrolysis, although there is some variability in the precise level at which this occurs.

A considerable proportion of the products of the primary degradation consists of a dextrin fraction, devoid of iodine coloration, which exhibits the following properties—reducing power by alkaline copper, 30–33 per cent $R_{maltose}$; rotation, $[\alpha]_D$, about 180°; molecular weight (from freezing-point), about 1000. These properties, reported by early investigators, suggest a *mean* chain length for the product of about 6 glucose units; there are indications, moreover, that certain of the specimens were obtained in a state of approximate chemical homogeneity. While a conclusion to this effect must be considered tentative, it seems significant that a consideration of the *achroic R value* leads to a similar view as to the molecular size of the primary degradation product (cf. p. 209).

With due reservations as to the exact chain length and the homogeneity of the product,[1] it may be concluded that α-malt-

[1] Even if the starch molecule ($n=30$) be transformed into 5 molecules of dextrin ($n=6$) it is conceivable that the fragment corresponding to the

amylase is endowed with a capacity for converting large fragments of the molecular structure of starch into dextrin molecules containing about 6 glucose units. The more complex amylodextrins and erythrodextrins described by early workers clearly represent larger residual portions of the original structure which are present before the completion of this process. Whether or not this "dextrinogenic" degradation proceeds from the end of the chain molecule is not deducible at present.

The second prolonged reaction phase which characterizes the progress of hydrolysis by this amylase clearly consists in some further cleavage of the product of the primary transformation. The reducing power rises slowly from 30 to 35 per cent of the theoretical maltose value to something of the order of 50 per cent (cf. p. 207). On the view that the primary product consists mainly of a dextrin of about 6 units, this limit of hydrolysis would indicate that the enzyme is unable to hydrolyse more than about one linkage in each dextrin molecule. The composition of the resulting smaller fragments remains uncertain, and it is not possible to suggest the precise character of this secondary process. Nor is it possible to decide whether the extreme slowness of the reaction is due to a low affinity of the enzyme for the primary product or to a low rate of decomposition of the enzyme-substrate compound formed in this case.

We are led to adopt the view that whereas α-malt-amylase rapidly degrades starch and large portions of the starch molecule, inducing their conversion into dextrin fragments containing something of the order of 6 glucose units, its further action on these primary products is relatively very slow. The great difference in the rates of these two processes, which must be considered to proceed to some extent concurrently, accounts for the relatively sharp inflexion in the progress curve when the primary "dextrinogenic" transformation has been completed.

PART V. OBSERVATIONS ON AMYLASES FROM OTHER SOURCES

Observations have been reported on amylase preparations from numerous plant and animal sources. In most cases, however, the object of investigations has been either to demonstrate the presence

aldehydic end of the starch molecule might retain the altered configuration which accounts for the low reducing power of starch itself. In this way only 4 out of the 5 molecules of dextrin might exhibit reactive aldehydic end-groups.

and degree of activity of starch-splitting enzymes of particular tissues, or to study the effects of varied reaction conditions (temperature, *p*H, salts, etc.) upon the rates of action of different amylases. Only in relatively few cases do the existing data permit even tentative conclusions to be drawn as to the nature of the processes of breakdown which are induced.

Owing to the relatively advanced state of knowledge concerning the saccharogenic and dextrinogenic components of malt amylase, these enzymes have come to be regarded as reference types for the comparison of other amylase preparations. The vast majority of extractible amylase systems are able to convert starch into products devoid of iodine coloration. It has long been recognized, however, that the ratio of the capacity to destroy the iodine colouring property and to induce the liberation of reducing groups varies considerably for preparations from different tissues. By analogy with the amylase system of malt, this fact is frequently held to indicate that many tissues contain two distinct amylase components, but that these are present in different proportions; in this way differences in the so-called dextrinogenic/saccharogenic ratios have been explained. While this current conception may receive confirmation from future investigations, it stands so far without experimental verification. Except in the maturing and germinating grains of cereals, no conclusive evidence is available to show the coexistence of distinct amylase components.

Dormant cereal grains have in the past been the only known tissues in which the saccharogenic (β) amylase alone is present in an active form, and there were grounds for regarding this as a biochemical oddity, dependent upon a differential inactivation of the dextrinogenic (α) component during the maturation of the fruit (cf. Ugrumov, 1935). More recently, however, observations reported by Giri (1934*a*, *b* and *c*) indicate that the amylase of the sweet potato tuber, *Ipomea batatas*, induces a closely similar process of starch degradation. Thus, starch hydrolysis by this enzyme ceases when the reducing power corresponds to 62 per cent of the theoretical maltose, and a resistant dextrin remains which exhibits a violet iodine coloration. By a modification of the Wijsman diffusion method (cf. p. 138) it was shown that the preparations contained no amylase analogous to the α-component of malt amylase. While certain observations are still required to establish the identity of the two enzymes (e.g. the nature and mutarotational behaviour of the reducing products) there is now little doubt of the occurrence in a state of isolation

of an enzyme closely similar to β-malt-amylase in the fleshy tissues of this tuber.

There is no conclusive evidence, as yet, that enzymes of the type of the dextrinogenic (α) component of malt exist in isolation in any tissue. The observations of Blom *et al.* (1936) indicate that "superclastase", a commercial preparation from bacteria, contains a closely similar enzyme (cf. Fig. 7B, p. 205). Since the preparations had been heated to 70°, and it is not stated whether or not the properties were altered, it cannot be concluded that this amylase alone was present initially.

Amongst amylases of animal origin, those of the pancreas and of saliva have been most investigated. There is a close similarity in the action of these two enzymes, but they do not induce identical breakdown processes.

In both cases it is clear that the substrate is transformed initially into reducing dextrins. Such early products from the action of ptyalin have been described by Köhler-Hollander (1934), and of pancreatic amylase by Freeman & Hopkins (1936*a*). The reducing power at the achroic point is 50–55 per cent of the theoretical maltose value during degradation by pancreatic amylase, and 40–45 per cent in the case of ptyalin. These are to be compared with the value of about 30 per cent which characterizes α-malt-amylase.

Starch hydrolysis by ptyalin ceases when reduction values corresponding to 75–83 per cent R_{maltose} are attained. With pancreatic amylase the limit of hydrolysis is lower, reported values varying from about 60 to 71 per cent (cf. Vonk & Braak, 1934; Freeman & Hopkins, 1936*c*; Hanes & Cattle, 1937). Although it is frequently assumed that maltose is the exclusive reducing product present at these advanced stages of degradation this is by no means rigidly established. On the contrary, the presence of reducing dextrins, which was first pointed out by Brown & Heron (1880) after the completed action of pancreatic amylase on starch, has been confirmed by Waldschmidt-Leitz & Reichel (1934) using erythroamylose and starch from glutinous rice as substrate. It is probable that maltose is the principal product formed by both amylases after prolonged action, but the presence of other short chain fragments is by no means excluded.

These two animal preparations exhibit certain general features in common with α-malt-amylase. In particular they appear to induce the initial cleavage of starch into dextrin fragments of reducing character and this is accompanied by the destruction of the iodine

colouring property. Whether or not the initial fragmentation is similar to that induced by α-malt-amylase is not clear, but whatever be the molecular size of the primary degradation products formed by the action of the two animal amylases, these are clearly degraded further with great rapidity. The termination of the primary "dextrinogenic" transformation of the substrate is accordingly not marked by any obvious slackening in the rate of hydrolysis, as is the case in degradation by α-malt-amylase. It thus seems probable that pancreatic and salivary amylases have higher affinities for short dextrin fragments (in relation to their affinities for complex products) than α-malt-amylase. (This might or might not be related to the presence of a second component, in addition to a dextrinogenic amylase, in the animal preparations.) This conception would provide a basis for explaining the general forms of the progress curves, the different *achroic R values* which characterize the three enzymes, and the fact that the *achroic R values* with ptyalin and pancreatic amylases rises with increasing substrate concentration (cf. p. 209). While this forms an attractive hypothesis, conclusive evidence in its favour is not available.

PART VI. THE MUTAROTATION OF THE PRODUCTS OF AMYLASES

An entirely separate line of investigation will now be considered which has been important in emphasizing fundamental differences in the degradation processes induced by different amylases.

R. Kuhn (1925) confirmed the early observation of Brown & Heron (1879) that when starch is rapidly degraded by malt amylase, the products exhibit rising (dextro-rotatory) mutarotation. This would be expected if maltose is first liberated in the β-configuration ($[\alpha]_D$, +118°), this form then reverting to the equilibrium mixture ($[\alpha]_D$, +138°) which consists of about 64 per cent β- and 36 per cent α-maltose.

Kuhn found, however, that the amylases of the pancreas and of *Aspergillus oryzae* gave products exhibiting falling mutarotation, indicating the reversion of products of the α-configuration to an equilibrium α-, β-mixture.

On the basis of this discovery Kuhn proposed the classification of amylases into α- and β-types, represented by the pancreatic and *Aspergillus* amylases on the one hand, and by malt amylase on the other, the two types being distinguishable by the fact that their

degradation products show respectively α- (falling) and β- (rising) mutarotation.

The two malt components

Ohlsson (1930) then observed that the isolated components of malt amylases were distinguishable by the direction of mutarotation of their products. The dextrinogenic malt amylase gave products which mutarotate downwards and this enzyme is accordingly of Kuhn's α-type. The saccharogenic amylase, on the other hand, showed the properties of the β-type amylase.

Accordingly, malt amylase is in reality a mixture of an α- and a β-type amylase and its earlier identification as a β-amylase is due to a preponderating effect of the saccharogenic component on the mutarotational behaviour of the products. In Table V are given examples of Ohlsson's observations on the two malt amylases, acting on electro-dialysed soluble starch substrate.

TABLE V. *Mutarotation of products of the two malt amylases* (from Ohlsson, 1930)

Saccharogenic component				Dextrinogenic component			
	Rotation				Rotation		
Time min.	Without soda	With soda	Mutarotation	Time min	Without soda	With soda	Mutarotation
8	2·63	2·68	+0·05	5	3·46	3·42	−0·04
25	2·38	2·46	+0·08	15	3·47	3·35	−0·12
40	2·26	2·37	+0·11	30	3·43	3·27	−0·16
90	2·19	2·27	+0·08	45	3·39	3·25	−0·14
120	2·20	2·27	+0·07	90	3·33	3·22	−0·11
300	2·25	2·25	0·00	210	3·21	3·18	−0·03

The general procedure for studying this aspect of amylase action is exemplified in these experiments. At intervals during the digestion samples are removed; to these is added anhydrous sodium carbonate which arrests the action of the enzyme and at the same time induces the rapid attainment of the mutarotational equilibrium. For comparison, the optical rotation is followed on a sample in which the degradation is allowed to proceed without interruption. After relatively long periods, the treatment with soda no longer induces changes in rotation (cf. Table V). This is due to the slow completion of the mutarotational transformation under the slightly acid conditions of the digest.

These observations have been confirmed by different authors, with preparations of the two malt enzymes separated in different

ways, and with a variety of different starch fractions and products as substrate.

Holmbergh (1933) reported that the dextrinogenic and saccharogenic amylases (separated by differential adsorption on rice starch, cf. p. 139) acting on soluble starch, gave products which exhibited mutarotations in agreement with Ohlsson's preparations.

Waldschmidt-Leitz & Reichel (1934) reported observations on a relatively simple dextrin which appears to represent a chain fragment containing 6 glucose units (cf. p. 213). By the action of the saccharogenic amylase (from barley) and the dextrinogenic amylase (separated from malt by the method involving the use of alumina $C\gamma$), products exhibiting β- and α-mutarotations respectively were formed.

Finally, Freeman & Hopkins (1936 *c*), using preparations of the two enzymes obtained from målt by the Ohlsson methods (p. 137) and, in addition, the saccharogenic component from ungerminated barley, reported concordant results when a number of different starch products were degraded. These included soluble starch, amyloamylose, erythroamylose, glycogen, and α-amylodextrin.

In a number of representative experiments, moreover, Freeman & Hopkins employed an ultrafiltration technique (using filters of ferric phosphate gel) by which it was possible to remove any unchanged substrate, and the more complex degradation products. In this way, filtrates containing only the smaller degradation products were obtained. In all cases such filtrates exhibited mutarotations in the appropriate directions.

From this considerable range of observations, which without exception are in agreement, it can be concluded with assurance that the dextrinogenic and saccharogenic malt amylases, irrespective of the particular starch fraction or product which is degraded, give rise to products which exhibit α- and β-mutarotation respectively. Furthermore, the observations of Freeman & Hopkins appear to exclude the possibility that these phenomena are due in the either case to mutarotation of unattacked substrate or of the more complex degradation products.

Kuhn's hypothesis

The original interpretation advanced by Kuhn involved two basic assumptions. First, the mutarotating product in all cases was considered to be maltose, which sugar was assumed to be liberated in the α- and β-forms by α- and β-amylases respectively. Second, the liberation of α- and β-maltose was assumed to result from the hydrolysis of α- and β-linkages in the starch molecule. This view was based on the fact that various other carbohydrases liberate sugars in the particular stereo-isomeric form in which they are originally linked.

Kuhn concluded, therefore, that both types of linkage are present in the starch molecule.

According to this hypothesis α- and β-glucosidic linkages were considered to occur alternately in a chain structure. The hydrolysis of any two succeeding β-linkages would yield the β-form of the intervening α-linked disaccharide; conversely, fission at succeeding α-linkages would liberate the α-form of a β-linked disaccharide. Since, however, no β-linked disaccharide accumulates, it followed that this must be a labile product which reverts to maltose. It was suggested that the instability was due to the presence of a furanose unit in this disaccharide.[1] As the logical outcome of his original assumptions, Kuhn was accordingly led to postulate, not only an alternation of α- and β-linkages, but also an alternation of pyranose and furanose units in the chain structure of starch.

Subsequent investigations, which have been discussed in Part I, exclude the possibility of the occurrence of either β-linkages or furanoside units in appreciable numbers in the starch structure. There is no reasonable doubt that the chain is built on the maltose pattern.

While Kuhn's theory of starch constitution is now of historical interest only, his primary assumption that maltose is the mutarotating product formed by both types of amylases requires more critical consideration than has generally been accorded it.

The hypothesis of Klinkenberg

A view of starch constitution advanced by Klinkenberg (1931; 1932*a*, *b*; 1934) was intended to explain not only the mutarotation of the products liberated by the α- and β-amylases of malt, but also the progress of starch degradation by these respective enzymes.

According to this hypothesis, starch contains two components, designated α-starch and β-starch, which constitute 36 and 64 per cent respectively of the total substance.

β-Starch is considered to be selectively attacked by β-malt-amylase, the hydrolysis of β-linkages (which are assumed to be present in this component) resulting in the liberation of β-maltose. When the transformation of this fraction into maltose is complete, the hydrolysis ceases, thus accounting for the limit of hydrolysis by this enzyme at the 64 per cent conversion stage. The residual material (erythro-

[1] It will be seen that on this theory neither the α-linked nor β-linked disaccharides liberated by the two types of amylase would be maltose. It was necessary to assume the production of labile disaccharides (different for the two amylase types) which in each case revert to maltose.

granulose or α-amylodextrin) is considered to represent the undegraded α-starch fraction.

α-Malt-amylase is assumed to hydrolyse selectively the α-starch fraction (36 per cent of the total), transforming it completely into maltose which is liberated in the α-form. In this way is explained the initial phase of rapid hydrolysis by this enzyme which comes to an end when the reducing power corresponds to 30–40 per cent of the theoretical maltose (cf. Fig. 7 A, p. 205). Since the reducing power continues to rise slowly after this stage, it is postulated that the residual undegraded β-starch (which is assumed to be present at this stage) undergoes a slow spontaneous transformation into α-starch, which is then degraded by α-malt-amylase as it is formed. (The reverse transformation, α-starch → β-starch, is considered not to occur —hence the sharp limit of hydrolysis exhibited by β-malt-amylase.)

According to Kuhn's theory the two forms of maltose were considered to arise by the hydrolysis of α- and β-linkages present in a single substrate; Klinkenberg postulates that they arise from the degradation of two distinct substrates.

α-Starch is considered by Klinkenberg to contain exclusively α-linkages, but no precise conception of the constitution of postulated β-starch fraction was advanced. Since, however, the hydrolysis of β-linkages in this fraction is assumed to liberate maltose, the assumption of the presence of alternating α- and β-linkages would seem inevitable. Since certain amylases (pancreas and *Aspergillus*) readily degrade at least 70 per cent of the starch substance (liberating α-mutarotating products) these would be acting upon part at least of the postulated β-starch fraction. It is clear, therefore, that the assumption of the origin of the two forms of maltose from different starch fractions does not circumvent the insuperable difficulties inherent in Kuhn's simpler hypothesis. The added assumption that α-linkages in a saccharide chain structure spontaneously revert to β-linkages cannot be considered seriously.

While these aspects of the hypothesis are untenable from purely chemical considerations, it must be stated that the main thesis of the selective action of the two malt amylases is not supported by the experimental evidence.[1] The value of Klinkenberg's theory lies in the interest it has aroused in the problems surrounding the malt amylases.

[1] The interpretation advanced by van Klinkenberg for the cessation of starch hydrolysis by β-amylase at the 60–66 per cent conversion stage is essentially similar to the view of Ling & Nanji (cf. p. 199). Thus the enzyme is considered to hydrolyse selectively one pre-existing starch fraction, leaving undegraded a second fraction.

The practically complete hydrolysis of amyloamylose by β-amylase seems the only fact which might be considered to support this view. As mentioned

The nature of the mutarotating products

In the case of the saccharogenic (β) amylase there is little doubt that the mutarotation is due to the liberation of maltose in the β-configuration, as postulated by Kuhn. Apart from the fact that the reducing products consist almost exclusively of maltose, Freeman & Hopkins (1936*c*) have recently demonstrated that the rate of mutarotation agrees closely with that of pure maltose under similar conditions.

As applied to α-type amylases, however, the assumption that the mutarotation is due exclusively to maltose has been clearly misleading, since it has created an impression that the reducing products consist solely of maltose.

The accuracy of the assumption that α-maltose is the principal mutarotating product during degradation by α-type amylases appears to have been first seriously challenged by Freeman & Hopkins (1936*c*). These authors, in agreement with earlier investigators, demonstrated that during the initial phase of degradation by α-malt-amylase, the reducing power is due mainly to reducing dextrins, maltose being present only in minimal amounts. Since α-mutarotation is observable almost from the beginning, it is clear that the mutarotating groups must be attached mainly to dextrin molecules.

Pancreatic amylase differs from α-malt-amylase in that no slackening in hydrolysis occurs after the conversion of the substrate into dextrins; these products are readily degraded further with the formation of increasing proportions of maltose. Thus, at an advanced stage of degradation (63·4 per cent apparent conversion), the velocity of mutarotation was found to be of the same order as that of maltose. During the early stages, however, the observations of these authors

earlier (p. 200), however, erythroamylose (the complementary fraction) is hydrolysable by β-amylase to about the same extent as unfractionated starch. Moreover, amyloamylose could not correspond with the postulated β-starch fraction, since it is readily degraded by α-malt-amylase, and pancreatic amylase.

α-Amylodextrin is regarded according to this interpretation as a preformed starch component. There is strong evidence to the contrary. In the first place, it is an *intermediate* member of a series of products which exhibit increasing resistance to the action of β-amylase (cf. pp. 198 and 228). In the second place, the chain length of 12 glucose units revealed by end-group assay establishes it to be a degradation product (cf. pp. 116 and 203).

The thesis that α-malt-amylase selectively transforms 36 per cent of the starch substance into maltose is patently untenable It appears to rest on a too-literal acceptance of the dictum that α-amylases produce α-maltose. It is clearly established that the bulk of the starch substance is transformed into reducing dextrins during the primary phase of the degradation (cf. p. 209).

showed the presence of reducing dextrins in considerable amount. It would appear, therefore, that in the case of the pancreatic amylase the mutarotation in the early stages is due to the liberation of α-forms of reducing dextrins[1] with some α-maltose, and in the later stages, mainly α-maltose.

There seems no doubt of the essential correctness of this interpretation of the mutarotation during the action of α-amylases. The proportion of the mutarotating groups attached to dextrins and to maltose must clearly vary both with the stage of degradation and with the particular amylase, but it is evident that in certain cases reducing dextrins constitute the principal mutarotating products.

The significance of the mutarotational phenomena

The three enzymes which have been shown to belong to Kuhn's α-type are pancreatic, *Aspergillus*, and α-malt-amylase. The relationship between the breakdown processes induced by these three enzymes is not established in detail, but it is clear that the preliminary degradation in each case involves the fragmentation of starch into dextrin molecules.

The liberation of α-mutarotating products accordingly seems related to this, the most common type of enzymic starch degradation. So far observations have not been reported for other dextrinogenic amylases (e.g. ptyalin), but it will be unexpected if these are found not to be of the α-type.

At present the single known example of Kuhn's β-class is the saccharogenic amylase of barley and malt. There is little doubt that this enzyme liberates maltose in the β-form. It seems equally certain, however, that the β-maltose is produced by the cleavage of an α-glucosidic linkage.

The adoption of an alternative view necessitates the postulation, not only of a widespread occurrence of β-linkages in the starch structure, but also of the liberation of labile products (containing presumably furanose units) which revert to the maltose configuration; neither of these assumptions can now be admitted as a possibility. Apart from the weight of chemical evidence which supports the α-glucopyranoside chain conception of starch structure, a range of observations indicates that the mutarotation of degradation products is determined by the enzyme rather than the particular starch product upon which it acts. Thus, even the simple 6-unit dextrin of

[1] The α- and β-forms of a dextrin will differ only in the configuration of the H and OH groups attached to C_1 of the aldehydic end-unit.

Waldschmidt-Leitz & Reichel, when degraded by α- and β-amylases, yielded products which exhibited α- and β-mutarotations respectively. The specific rotation of this product excludes the possibility that two out of the five linkages are of the β-type (cf. p. 213).

It must be concluded, therefore, that the production of β-maltose by the saccharogenic amylase reflects a peculiarity of the manner in which the hydrolytic cleavage of α-linkages is induced by this enzyme. If, as seems probable, the degradation proceeds from the non-aldehydic end of the chain, it must be assumed that a Walden inversion occurs at the C_1 grouping of the maltose fragment during the fission.[1] There are no chemical objections to this interpretation, and objections on enzymic grounds rest entirely on the fact that so far no parallel examples have been encountered amongst enzymes which hydrolyse simpler carbohydrates.

PART VII. GENERAL DISCUSSION

ENZYMIC DEGRADATION IN RELATION TO THE CONSTITUTION OF STARCH

A number of specific features characterizing the action of different amylases seem clearly discernible, and it is proposed now to consider to what extent these conceptions of enzymic processes of starch degradation are in harmony with, and impinge upon, the view of the constitution of starch which has arisen from the purely chemical investigations outlined in Part I.

This constitutional theory may be summarized briefly as follows:

(1) The starch substance is considered to be homogeneous chemically, in the sense that it consists of uniform chemical units or macromolecules.

(2) The macromolecule is conceived to be an unbranched chain structure containing about 30 glucopyranose units, joined together by (1–4) glucosidic linkages as in maltose. The molecular weight would accordingly be about 5000.

(3) The macromolecules do not normally exist as free individuals but they are associated together to form colloidal aggregates of high dimensions. The mechanism of this association, and the nature of the linkages involved, remain obscure.

[1] If the aldehydic end were involved, it might be suggested that the enzyme combines only with chain molecules terminated by aldehydic groups of the β-form; the terminal maltose fragment would then be set free in the β-configuration.

(4) Owing to differences in the degree (and possibly in the kind) of molecular aggregation of the basic chemical units, starch may exist in different physical states. Physically distinguishable starch fractions, such as amylose and amylopectin, are thus held to be composed of identical macromolecules.

(5) The reducing power of starch is considerably less than would be expected for a chain of 30 glucose units terminated by a free aldehydic group. It has been suggested that the aldehydic end-unit may take part in the linkages between macromolecules, which are responsible for the phenomenon of molecular aggregation.[1]

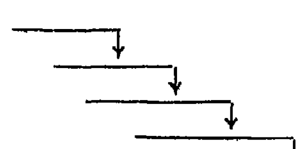
In considering the established facts of amylase action in relation to this chemical conception of starch, the discussion may justifiably be restricted to the separable component enzymes of the malt amylase system. These appear to constitute the most extreme types of starch-splitting enzyme recognizable at present; moreover, the characterization of the action of other amylases is far less complete.

The starch-liquefying amylase (*amylophosphatase*)

From the point of view of starch constitution, the considerable interest which attaches to this enzyme lies in the fact that it appears to attack selectively the bonds which are involved in the formation of molecular aggregations.

[1] *Note added May* 24. The formulation first suggested for xylan (cf. p. 129) has more recently been considered in relation to glycogen and starch. Haworth, Hirst and Isherwood (1937) report that after exhaustive methylation of glycogen, dimethyl glucose is formed on hydrolysis in an amount approximately equivalent to that of the terminal tetramethyl glucose unit. It seems likely therefore that one glucose unit in the macromolecule contains only two free hydroxyl groups, this unit being joined at three points with adjacent glucose units.

On the interpretation suggested in the xylan paper, glycogen would be represented by a number of macromolecules (containing 12 or 18 α-glucopyranose units) which are themselves joined by union of the reducing end of each chain with a hydroxyl of an adjoining chain. This postulated union might involve either a glucosidic bond or a hydroxyl (co-ordinated) bond. Thus, if the individual macromolecule be represented as $\rightarrow$, the arrow head indicating the aldehydic end of the chain, this conception of the linking of consecutive macromolecules may be expressed diagrammatically in the following "laminated formula":



It would be premature to discuss this conception in relation to the main subject under consideration. Certain possible implications will become evident below (cf. p. 229 and Fig. 10, p. 234).

It would be premature to conclude from the existing data that the disaggregated product of the action of this enzyme represents starch in the form of its individual macromolecules, but the properties suggest that this may possibly be the case. The reducing power is particularly suggestive, since on a stoichiometric basis it would indicate a chain length of 30–40 units.

The importance of this possibility emphasizes the need for a rigid characterization of this enzymically disaggregated starch, particularly by the method of end-group assay and by determinations of osmotic pressure.

As has been made clear, it is by no means certain that this enzyme hydrolyses exclusively phosphoric acid linkages present in starch, and the desirability of extending the observations to include starch products which contain no phosphorus has been discussed. On the other hand, the reported parallelism between viscosity change and phosphorus cleavage in the case of potato starch, is of obvious importance in suggesting that phosphorus may function in some way as a "centre of aggregation" for the molecular chains.

The saccharogenic (β) amylase of barley and malt

There is little doubt that this amylase attacks the chain molecule of starch at one end, and that the degradation process consists in the liberation of successive terminal maltose fragments. It is probable, although not conclusively established, that the non-aldehydic end of the chain structure is involved in this process.

A consideration of the different established features of this amylase, in relation to the conception of starch as a chain of recurrent α-glucose units joined by linkages of the maltose type, leads to a rather definite view of the enzyme-substrate relationship.

Thus, the peculiarly end-wise nature of the process of degradation points to a combination of the enzyme with the terminal glucose unit. Since maltose is not hydrolysed, the enzyme is clearly incapable of hydrolysing the linkage immediately adjacent to the terminal unit. The exclusive production of maltose during the degradation of starch establishes, in fact, that the enzyme induces the hydrolysis only of the linkage between the second and third glucose units in the chain structure.

The simplest conception of the enzyme-substrate relationship would appear to be that the enzyme comprises two distinct parts. One of these combines with some grouping in the terminal glucose

unit[1] and this enables the second part of the enzyme to approach and induce the cleavage of the second linkage from the end of the molecular chain. The distance between the postulated parts of the enzyme would be of the order of the length of a maltose molecule, that is about 10 A. units.

The only alternatives to this "lock-and-key" conception of the relationship appear to require the assumptions either that the second linkage from the end is for some obscure reason relatively unstable and so more readily split than other linkages, or that the mere combination of the enzyme with the terminal glucose unit renders it unstable.

If, as now appears likely, the chain structure is degraded from the non-aldehydic end, it must be concluded that in inducing the cleavage of the linkage between the second and third units, the enzyme induces at the same time a Walden inversion of the newly liberated aldehydic group. There are no chemical grounds for considering improbable the formation of β-maltose in this manner. As has been made clear earlier, the alternative view that the liberation of β-maltose could arise only by the hydrolysis of a pre-existing β-linkage rests upon the application of a purely empirical rule; it leads, moreover, to constitutional conceptions which appear entirely untenable.

Certain broader features of the action of this amylase are of considerable interest from the point of view of starch constitution and, in particular, the limit of the hydrolytic action of this enzyme. After the transformation of about 50 per cent of the starch substance into maltose, the reaction proceeds only slowly. Under acid conditions (*p*H 3·4), the hydrolysis ceases sharply at the stage of 53 per cent conversion; under other conditions (e.g. at *p*H 4·6, or more alkaline reactions) limits of hydrolysis varying from 60 to 67 per cent are observed.

It is to be noted that, on the basis of a chain length of 30 glucose units for the starch chain molecule, the removal of 8, 9 and 10 maltose fragments would correspond, respectively, with 53, 60 and 66·6 per cent transformations of the substrate. The almost exact agreement of these values with the different observed limits of hydrolysis by this enzyme suggests that the value of 30 units (rather than 25 or 35, for example) represents the chain length of starch, as was pointed out by Blom *et al.* (1936).

The chain lengths of the residual amyloid dextrins at the stages of

[1] If, as seems probable, the non-aldehydic end-unit is involved, it is tempting to suggest the hydroxylated C_4 position as the grouping which combines with the enzyme.

53 and 60 per cent conversion would be 14 and 12 glucose units respectively, the latter product representing the resistant α-amylodextrin. Here again, the reported value of 12 units for α-amylodextrin as revealed by end-group assay supports the value of 30 units for starch itself.

While these features of the degradation process provide important support for the view that the macromolecule contains about 30 glucose units, it is evident that present constitutional theories provide no clear explanation for the cessation of the action of the enzyme at these well-defined stages. If the chain molecule presented a perfectly regular configuration pattern over its entire length this phenomenon would not be expected to occur. It must be assumed, therefore, that the enzyme encounters some structural alteration in the neighbourhood of the 16th to 18th glucose units of the chain. It seems likely, moreover, from the observations which have been considered (e.g. the effects of autoclaving the resistant amyloid dextrins) that obstructions to the action of the enzyme occur at intervals in the remaining length of the macromolecule. The conclusion seems inescapable that that portion of the starch molecule which gives rise to the resistant amyloid dextrins must contain configurational modifications which prevent the effective combination with β-malt-amylase. Such modifications need not necessarily involve true chemical linkages, since attachments by light valency forces might conceivably destroy the specificity requirements of the enzyme.

The precise nature of the modifications in this portion of the macromolecule remains largely a subject for conjecture. It is tempting to suggest that the resistance to the action of the enzyme is connected in some way with the association of the macromolecules into aggregated structures. Thus, throughout the process, whether the hydrolysis terminates at the α-amylodextrin stage or whether further degradation is induced by resort to autoclaving, the residual resistant portions of the structure exist in an aggregated state; the resistance exhibited by these products stands in contrast to the ease with which non-retrograding dextrins of corresponding molecular magnitude are degraded by β-amylase.

According to this view, one is led to postulate that the portion of the starch molecule which is readily degraded by β-amylase is not involved in the formation of molecular aggregations, but that only the terminal portion of the structure, comprising apparently some 12 glucose units at the aldehydic end of the molecule, takes part in the formation of these associations.

It seems probable that further investigations of amyloamylose will elucidate these still-obscure constitutional features (cf. p. 200). The determination of the chain length of this product will be of particular interest since this will indicate whether or not it is composed of the macromolecules, containing about 30 glucose units, which appear to characterize all other fractions and varieties of undegraded starch which have so far been examined. If this is found to be the case, it will be clear that the macromolecules are capable of existing in a form which is almost free from the structural features which normally bring about the cessation of the action of β-amylase at 60 per cent conversion.

Finally, it may be pointed out that the existence of a resistant portion of the starch molecule is indicated clearly by the observations on various amylases, in addition to the saccharogenic (β) amylase of barley. This is quite clear, for example, in the case of the salivary and pancreatic amylases. Owing to the relatively simple nature of the degradation induced by β-amylase, however, further analysis of the action of this enzyme appears at the present time to offer the greatest hope of solving these aspects of the constitutional problem.

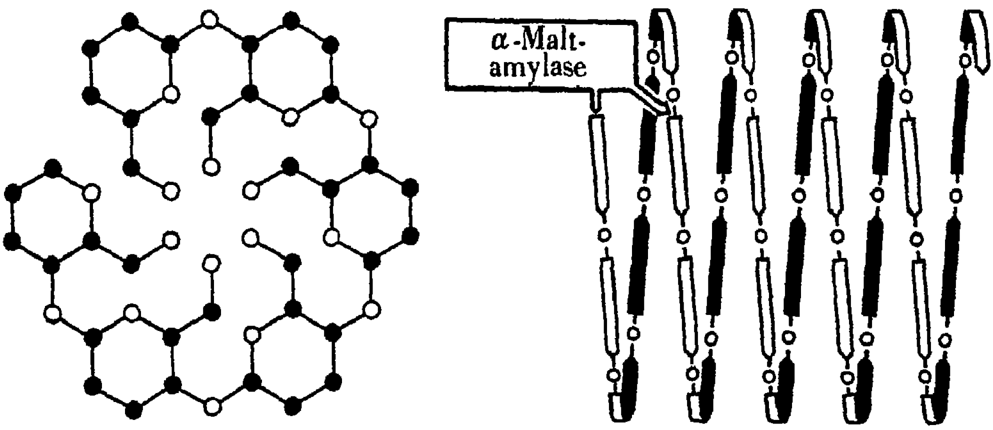
The dextrinogenic (α) amylase of malt

The feature of this amylase which invites consideration from the point of view of starch constitution is the fact that it readily induces the cleavage of undegraded starch and of complex portions of the starch molecule into reducing dextrins, but exhibits only to a small degree a capacity to further degrade these products. This highly preferential attack of linkages in more complex substrates suggests that relatively long portions of the chain structure are involved in the formation of the enzyme-substrate complex during the rapid primary phase of starch degradation by this amylase.

The simplest basis for interpreting this process seems to be afforded by assuming again, as in the case of β-malt-amylase, that two distinct parts of the enzyme react with different groupings in the substrate. It is necessary to postulate, however, that the two groupings capable of combining with α-malt-amylase must be separated by a considerable number of glucose units in the chain structure. The intervening chain fragment is then liberated by the induction of hydrolysis in the vicinity of one or of both points of attachment, depending on whether the terminal portion or a non-terminal portion of the substrate molecule is involved in this reaction.

According to this view, the conversion of starch into relatively complex reducing dextrins is attributed to the spacing between two "points of attachment" between enzyme and substrate. The objection immediately arises that in this hypothesis an unlikely degree of structural organization of the enzyme appears to be assumed.

The possible interest in this formulation, however, lies in the suggestion, derived from early investigations, that dextrin molecules containing about 6 glucose units are formed in considerable amounts during this transformation. Indeed, on this basis, a simple and plausible interpretation of the enzyme-substrate relationship can be suggested.



A B

Fig. 9. A, hexagonal arrangement of 6 α-linked glucopyranose units (Sachse conformation) modified after Haworth (1929). B, hypothetical spiral model of α-linked chain of 30 glucose units, showing possible mode of combination with α-malt-amylase.

Thus, before the establishment of the open chain character of the starch molecule, Haworth (1929) had drawn attention to the fact that the union by α (1–4) linkages of 6 glucopyranose units of the Sachse configuration might be expected to give rise to a strainless hexagonal ring structure of remarkable structural symmetry (Fig. 9 A). Extension of this conception to longer chains of α-glucopyranose units suggests that these might exist in the form of close spirals, each coil of which would contain exactly 6 glucose units (Fig. 9 B). It is evident that the representation of the chain molecule in this spiral conformation provides a ready basis for interpreting the cleavage of the structure into fragments containing 6 glucose units, since linkages separated by this number of units are brought into lateral proximity. According to this view, the two groupings in the substrate which form the points of attachment for α-malt-amylase would be considered to be present in glucose units occupying corresponding positions in

successive coils of the spiral. If, for example, the degradation proceeds from the end of the molecule, the enzyme would be pictured as combining with some part of the terminal glucose unit and simultaneously with a grouping in the vicinity of the linkage between the 6th and 7th units from this end of the chain structure (Fig. 9 B). With the induction of cleavage at this point one complete coil of the spiral would be liberated, yielding a 6-unit dextrin fragment, terminated by a reactive aldehydic group, and presenting the configuration shown in Fig. 9 A.

This speculative conception of a spirally arranged molecular chain is of some interest in relation to the iodine colouring property of starch dextrins. Thus, the available evidence suggests that fragments containing as many as 6 (or possibly 7) glucose units give no coloration with iodine, whereas longer fragments exhibit this property. It might accordingly be suggested that the formation of coloured iodine compounds depends in some way upon the presence in a dextrin molecule of more than one complete coil of the spiral. The postulation of some such configurational basis of the iodine colouring property gains plausibility in view of the otherwise inexplicable fact that α-malt-amylase induces the conversion of starch into achroic dextrins with what appears to be the smallest possible number of hydrolytic cleavages. The spiral model would thus provide a common basis for explaining the mode of attack by this amylase, and the fact that the process approximates to an ideally "dextrinogenic" transformation (cf. p. 208).

It may be pointed out that there are other grounds for suspecting that the units of α-glucopyranose chain structures may be arranged in some way in groups or multiples of six. There is, for example, the puzzling fact that the two known forms of glycogen contain 12 and 18 glucose units respectively, and that so far no specimens of intermediate chain lengths have been encountered. Under the action of β-malt-amylase on starch, moreover, it is clear that 18 glucose units are normally removed (in the form of maltose) and that a residual portion of the molecule containing 12 units is left intact.

The adoption of the conception of a spiral chain conformation on the lines suggested above would clearly offer a rudimentary basis for the interpretation of these facts. The two forms of glycogen would then be represented as structures containing, respectively, two and three complete coils of 6 units. The starch molecule would be conceived as consisting of a five-coiled spiral, of which three coils are

readily degraded into maltose under the action of β-amylase, and the remaining two coils (corresponding to the α-amylo-dextrin portion) are resistant to this enzyme, due possibly to the participation of this portion of starch molecule in the formation of molecular associations, as has been suggested above.

This hypothesis is clearly speculative to a high degree, but the consideration of such a formulation at the present time serves a two-fold object. In the first place, it demonstrates that the peculiarities of the process of starch breakdown under the action of α-malt-amylase do not necessarily require the postulation of different types of linkages in the starch molecule. (It has been suggested, for example, that certain groupings in the starch structure are responsible for the iodine colouring property and that these are specifically attacked by this amylase.) In the second place, the importance of the constitutional issues which are raised in this formulation emphasizes the desirability of a rigid characterization of the products of the primary degradation induced by this amylase, and by other amylases of the dextrinogenic type.

Summary

From this survey it will be clear that each of the separable components of the malt amylase system presents aspects of great interest from the point of view of the Haworth-Hirst theory of the constitution of starch. The breakdown processes induced by these different enzymes, so far as their nature is now discernible, display no features which appear definitely incompatible with this view of starch structure; indeed, certain aspects of the theory receive important substantiation from enzymic data. It is evident, however, that a number of facts regarding the action of amylases find as yet no basis for precise interpretation in terms of the constitution of the substrate. In the present state of knowledge this is not surprising. Chief amongst these issues is the indisputable fact that, so far as susceptibility to attack by amylases is concerned, the molecular chain structure is not uniform over its entire length. Whether this is due to the participation of one end of the macromolecule in the formation of molecular aggregations, as has been suggested as a possibility, or whether configurational irregularities of a still unsuspected nature occur in the structure, constitute perhaps the important questions which are raised. In any event, finality is not to be expected until the intimate nature of the associations of macromolecules becomes established, and it seems likely that progress

toward the solution of this intricate problem will depend to some extent upon the use of enzymes as selective hydrolytic agents.

In Fig. 10 is presented diagrammatically the manner in which the three components of malt amylase might be conceived to attack the starch structure. The substrate is represented by three macromolecules of starch, each containing 30 glucose units. The terminal portions, comprising 12 glucose units at the aldehydic end of each macromolecule, are involved in linkages of some unspecified nature which unite the macromolecules into an aggregated structure, and which involve the terminal reducing groups.

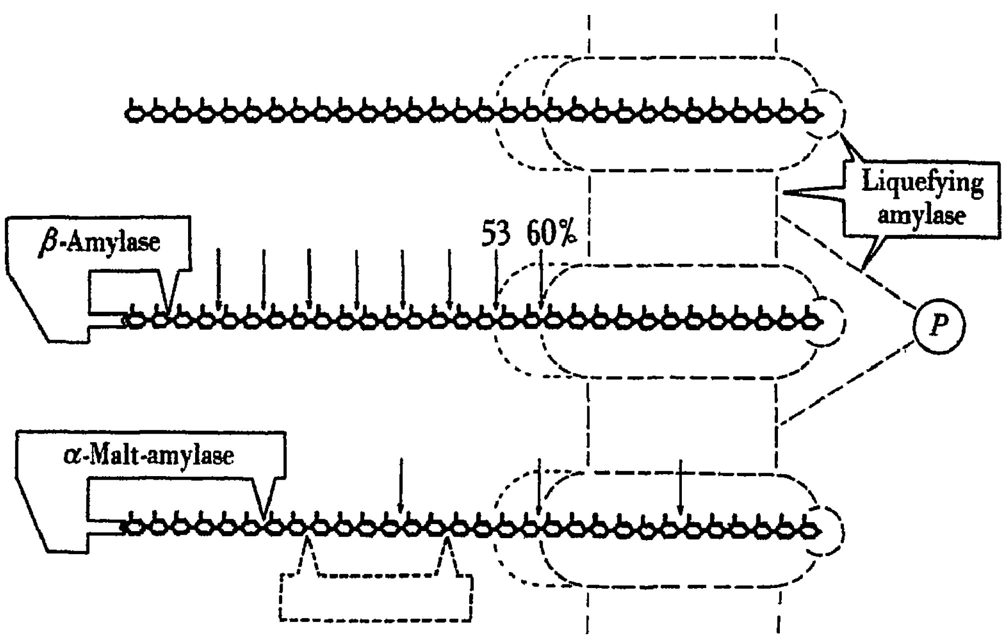


FIG. 10. Hypothetical schema of the mode of action of amylases in relation to the constitution of starch.

The liquefying amylase is pictured as inducing the rupture of the bonds uniting different macromolecules, the process being accompanied by the cleavage of phosphorus and the transformation of the terminal aldehydic groups into a reducing form.

The saccharogenic (β) amylase is represented in combination with a macromolecule at its non-aldehydic end. The enzyme-substrate compound is shown as involving the union of distinct parts of the enzyme with the terminal glucose unit and with a grouping near the second linkage from this unit, at which point cleavage is induced. A continuation of this process results in the liberation of successive disaccharide fragments (by hydrolysis of the linkages indicated by arrows in the diagram) until the enzyme encounters some modification in the structure in the vicinity of the 16th to the 18th glucose units (corresponding to 53 and 60 per cent conversion of the sub-

strate into maltose). Degradation to this extent is conceived as proceeding without inducing the disruption of the aggregated condition of the residual portions of the molecules which correspond to the resistant α-amylodextrin.

Finally the dextrinogenic (α) amylase is pictured attacking another of the macromolecules. The two parts of this enzyme are united with the terminal glucose unit and with a grouping in the vicinity of the link between the 6th and 7th chain units (cf. Fig. 9 B). Cleavage at this point is conceived as resulting in the liberation of a reducing dextrin molecule and subsequent points of hydrolysis are indicated. An alternative mode of attack by this enzyme is indicated in ghost form.

It will be clear that this diagram incorporates features which range from relatively well-established to admittedly speculative hypotheses. It is accordingly put forward at the present time, not as an expression of opinion, but rather as an outline of the problems which now appear to be of outstanding importance in this complex field of investigation.

THE BEARING ON PROBLEMS OF STARCH METABOLISM IN THE PLANT

Appreciable advances have been made in recent years in the direction of clarifying the problems of the chemical constitution of starch and the manner of its degradation *in vitro* under the action of different amylases. Continuing progress of knowledge in this direction may be expected to elucidate the obscure questions associated with the metabolism of starch in the living plant; so far, however, the conceptions which have emerged appear to bear only indirectly upon the physiological aspects of the starch problem.

Constitutional studies on starch are proceeding inevitably from finer to grosser structures—from the individual glucose unit and its mode of union, to the chain molecule, and finally to the still obscure question of the manner in which these larger units are bound together to form highly colloidal structures. The point has not yet been reached when this line of investigation throws light on the chemical organization of the native starch grain. Accordingly, no new basis is afforded for interpreting the outstanding biological fact that starch is known in nature only in the form of starch grains, and that these bodies are formed exclusively within the confines of specialized cellular organs, the plastids of the plant cell.

This peculiar localization of starch within the plastid in an insoluble form, raises questions of basic physiological significance. It may be that the plastid represents merely the site at which some, as yet unrecognized, form of dissolved starch is transformed reversibly into the substance of the starch grain; on the other hand, the whole range of metabolic reactions, including synthesis of starch from sugars, deposition in the visible grain form, and subsequent reconversion into sugars, may be localized within the plastids. Whichever alternative may prove to be correct, there is little doubt that the obscure mechanisms or conditions which occur within the plastid must play an important part in the so-called "starch-sugar" balance of plant tissues.

Extensive observations have been made on the effects of different factors (such as temperature, sugar concentration, inorganic salts, water content and light) on the starch$\rightleftharpoons$sugar transformations in the tissues of different plants. One main feature which has emerged from this field of investigation deserves comment here.

Thus, the products which accumulate when starch is hydrolysed within the living plant are usually mixtures of sucrose, glucose and fructose. It is a striking fact that maltose, and dextrins, occur but rarely, and only in small amounts, in plant tissues. There is thus no doubt that the degradation of starch under the action of extracted amylases gives rise to products which are different from those which accumulate when starch is degraded in the living cell.

This fact has been considered by some investigators to favour the view that the glucose units in the starch molecule pre-exist in the "active" or glucofuranose form, and that maltose represents the product of a reversion-synthesis from some labile intermediary. On this point, however, the chemical evidence seems conclusive in showing that the glucose units, as they pre-exist in starch, are of the stable pyranose configuration.

It seems clear, therefore, that the generation of fructose units (whether free fructopyranose, or fructofuranose combined in sucrose) which is normally observed when starch is degraded in the living cell must be due to the operation of a system which facilitates the conversion of the glucopyranose units of the polysaccharide into fructose units, either during the actual process of degradation or subsequent to it. The nature of this system, and its possible relationship with the extractible starch-splitting enzymes, clearly forms one of the central problems in the field of the carbohydrate metabolism of plants.

A strong suggestion as to the type of mechanism which may be

involved comes from the conceptions which are now emerging in connexion with the breakdown of glycogen (and starch) by the enzyme systems of muscle.

Thus, it has long been known that the glycolytic system of muscle is able to produce lactic acid much more rapidly from these polysaccharides, than from glucose or fructose. It is possible, moreover, to obtain muscle preparations which are incapable of acting upon the free hexoses, but which retain the capacity to produce lactic acid from glycogen, starch or hexosediphosphate (cf. Laquer & Meyer, 1923). More recently, it has been established that hexosediphosphate is formed as an intermediary during the conversion of glycogen to lactic acid (Parnas & Baranowski, 1935). It appears probable therefore that the degradation of the polysaccharide by this system consists in the detachment of the individual glycopyranose units, accompanied by (or possibly as a result of) the introduction of phosphoric acid residues, and that the product is liberated in the form of an esterified fructofuranose molecule. Whether or not the amylase system of muscle plays any part in this process is not so far established.

The muscle system affords a clear-cut example of an enzymic mechanism by which α-glucopyranoside chain molecules are degraded to yield a derivative of fructofuranose. Apart from the remarkable configurational transformation which is thus readily induced, the fact deserves emphasis that the 1 : 6-diphosphoric ester of fructofuranose which is liberated in this process, constitutes an ideal example of a sugar form which is mutable under biological conditions. This ester, for example, under the action of various phosphatases, gives rise to an equilibrium mixture of the 6-monophosphoric esters of glucose and fructose (fructofuranose). Alternatively, under the action of the so-called zymohexase, hexosediphosphate is transformed rapidly and reversibly into two molecules of triosemonophosphate.

There is at present no direct evidence to indicate that comparable enzymic mechanisms are operative during the degradation of starch in plant tissues. Since, however, the extractable amylases do not represent the intact starch-degrading system of the living cell, and since it is clear that analogous transmutations of sugars must occur in the plant, investigations in this direction would appear to offer some prospects of elucidating the problems of the metabolism of starch in the plant.

The foregoing article constitutes by no means an exhaustive survey of the vast literature bearing on the subjects of the chemical consti-

tution of starch and the manner in which it is degraded under the action of amylases. It is inevitable that many observations, some perhaps of great significance, have received no mention. The object throughout has been to outline the features in these interrelated fields which now seem clearly established, and to formulate as precisely as possible the main problems which are now discernible. The scope of the discussion may be summarized conveniently by the inclusion of a Table of Contents.

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Finally, I have pleasure in recording my indebtedness to Dr F. F. Blackman, F.R.S., and Mr G. E. Briggs, F.R.S., at whose suggestion I began work in this field, and who have shown continued interest in its progress.

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A REVIEW OF RECENT WORK ON THE NITROGEN METABOLISM OF PLANTS, PART II

By H. S. McKEE
Department of Botany, Oxford

(With 1 figure in the text)

The acid amides

In her 1929 review M. E. Robinson summarized the work of E. Schulze and of Pryanishnikov on the role of the acid amides, especially asparagine, in the plant's protein metabolism. Schulze held that asparagine was an essential stage in the synthesis of protein, while Pryanishnikov regarded it rather as a means of storing ammonia in a harmless form, its function thus being similar to that of urea in the animal, except that the latter is excreted and the nitrogen of the asparagine is still available for protein synthesis. The analogy of function between asparagine and urea has also been stressed by Murneek (1935) in a review of work on the metabolism of amides in the plant. The schemes he gives for their formation must now be regarded as unlikely, but this, of course, does not affect the functional analogy. Pryanishnikov's views on the position of asparagine in plant metabolism may be indicated in the following diagram, which is quoted from M. E. Robinson.

$$\begin{tikzcd}
 & \text{Amino-acids} \arrow[r] & \text{Protein} \arrow[r] & \text{Amino-acids} \arrow[d] \\
HNO_3 \arrow[r] & NH_3 \arrow[u] & \text{Asparagine} \arrow[l] & NH_3 \arrow[l]
\end{tikzcd}$$

Since then the most important work in this field has been that of Mothes (1926, 1929, 1931). He found that amides accumulate in detached leaves in the dark, that this accumulation is accelerated by increased temperature, and that leaves which have lost much of their carbohydrate through prolonged darkening accumulate ammonia instead of amides. Detached leaves which are not too old do not accumulate amides in the light nor if they are supplied with glucose in the dark. The amides and ammonia arise as a result of protein breakdown, but their formation is a secondary process dependent on the presence of oxygen. In the presence of narcotics and in autolysis

there is no amide formation, but an accumulation of amino-acids and later of ammonia. Young leaves with abundant carbohydrate synthesize protein when supplied with ammonium salts, those with less carbohydrate store the ammonia in the form of asparagine, while those with little or no free carbohydrate accumulate free ammonia till they die of ammonia poisoning. Mothes regards the chief role of amides in the plant as the rendering harmless of ammonia and in the main his views resemble those of Pryanishnikov.

Fife & Frampton (1935) have found that when sugar-beet leaves are exposed to high concentrations of carbon dioxide the reaction of the cell-sap becomes alkaline and not, as might have been expected, acid. This result was found to be mainly due to the reaction

$$R.\mathrm{CONH_2}+2\mathrm{H_2O}=R.\mathrm{COOH}+\mathrm{NH_4^+}+\mathrm{OH'}.$$

When the treated plants were allowed to recover the reaction returned to its original value.

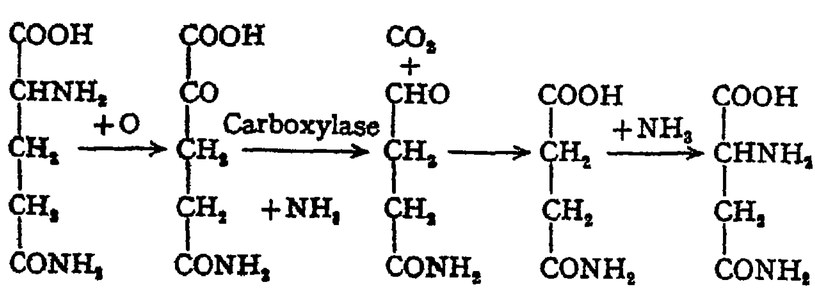
$$R.\mathrm{COOH}+\mathrm{NH_4^+}=R.\mathrm{CONH_2}+\mathrm{H_2O}+\mathrm{H^+}.$$

These results suggest the possibility that the formation of amides may in some circumstances be affected by the carbon dioxide produced in respiration. The sudden rapid production of ammonia by leaves which have been starved in the dark until they have lost all their available carbohydrate may conceivably be due to the carbon dioxide concentration inside the cell having passed some definite limit. It is more probable, however, that the utilization of nitrogenous substances in respiration, due to the shortage of carbohydrates, has led to the production of ammonia, which has been stored as amides while sufficient non-nitrogenous precursors for their formation are available, and that subsequent accumulation of free ammonia has led to the death of the cells and autolytic breakdown of the accumulated amides.

Vickery *et al.* (1933) have studied the changes in detached tobacco leaves during curing. In this process the leaves are kept without a supply of water and the temperature is allowed to fluctuate. Similar experiments were also done with detached leaves whose petioles were dipped in water. The following description refers to the latter experiments, as they are the more comparable with those, to be described later, of Yemm (1937) and myself. During the 300 hours which the experiment lasted half the protein originally present was broken down to amino-acids, which did not accumulate as such, but were converted into amides. During the first 64 hours nitrates increased

by 65 per cent, a somewhat unexpected result for which there is no obvious explanation. Protein was broken down slowly at first, when there was still a supply of available carbohydrate, and at a higher and approximately constant rate later. Amides began to accumulate at 120 hours, and up to 183 hours the amide formed was relatively resistant to acid hydrolysis and was probably asparagine. Later an unstable amide, hydrolysable by boiling at *p*H 3, was formed. The authors do not name it, but it was probably glutamine, which has since been found in the tobacco plant. Free ammonia began to increase about the same time as the unstable amide, but it did not reach a high level, and there was no serious breakdown of amides during the experiment.

Similar experiments with detached barley leaves have been made by Yemm (1937) and by myself (unpublished). The general sequence of events is similar to that found by Vickery *et al.* (1933), but the tempo is much more rapid, as is the loss of available carbohydrates. Glutamine and asparagine were estimated separately, using the method of Chibnall & Westall (1932) with slight modifications. It is possible that the values for glutamine include glutamyl peptides, for it has been shown by Melville (1935) that some such peptides are so readily hydrolysed as to be estimated by the methods for glutamine. Glutamine, asparagine and ammonia, in that order, rise to maxima, the first two falling again later. While the glutamine content is falling that of asparagine rises, so that the total amide content remains steady for a time. It is not easy to assess the significance of this result. A direct formation of asparagine from glutamine need involve only biochemically possible reactions (III). Or the relation may only



......(III)

be indirect, with the ammonia produced by the breakdown of glutamine going to form asparagine and the carbon residue being further broken down. After about 130 hours, the actual time varying somewhat from sample to sample, the amides break down suddenly to liberate large quantities of ammonia. Immediately before this breakdown

there is, in contrast to the results of Vickery *et al.* (1933) with tobacco, much more asparagine present than glutamine (Fig. 1). There is some evidence that this ammonia is formed by processes due to the leaves themselves, in which case it is the proximate cause of their death rather than a post-mortem phenomenon, but it is difficult to establish this point definitely, as in the final stages bacterial sterility is virtually unobtainable.

Glutamine is frequently found, as in these detached barley leaves, in smaller quantities than asparagine, which has given rise to the idea

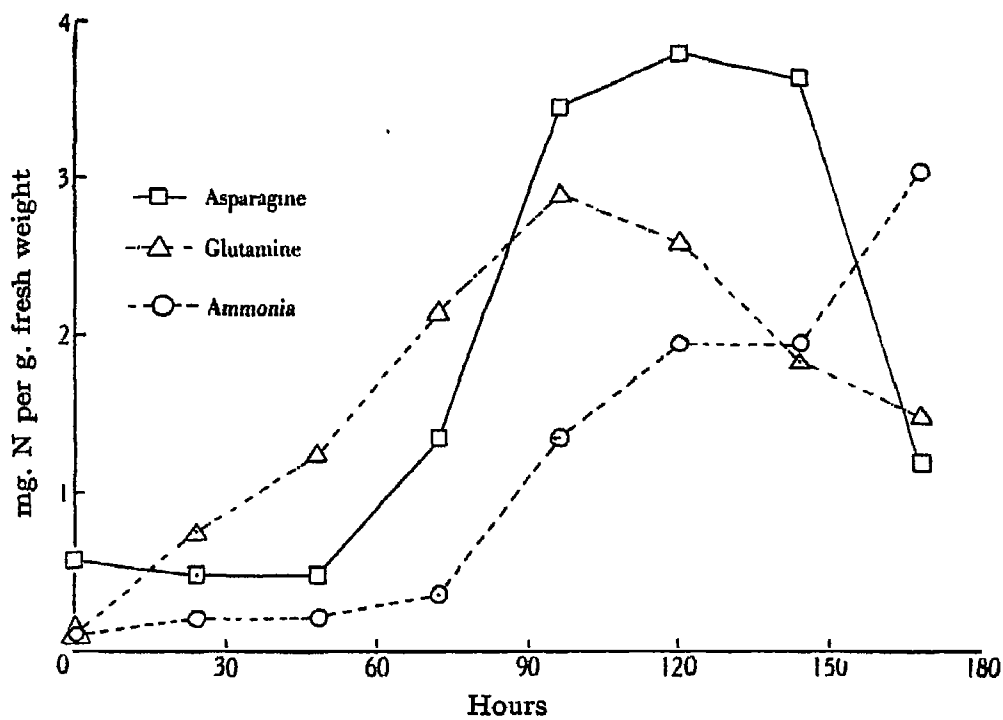


Fig. 1. Change in N-fractions in detached leaves from plants supplied with nitrate.

that it is directly formed by the hydrolysis of protein but that asparagine is formed indirectly from the products of protein breakdown. This is particularly plausible in the Gramineae, whose seed proteins yield on hydrolysis large quantities of glutamic acid and ammonia, which (Damodaran *et al.* 1932) are probably derived from amide units in the protein molecule. Nuccorini (1930), from a study of the amounts of glutamic and aspartic acids obtained from the seed proteins of *Ricinus*, came to the conclusion that in the seedlings glutamine is a direct product of protein hydrolysis, but that asparagine is formed indirectly through amino-acids. It is clear, however, that in many plants, such as tomato and sugar-beet, the amounts of glutamine found are too great to have arisen solely in protein breakdown.

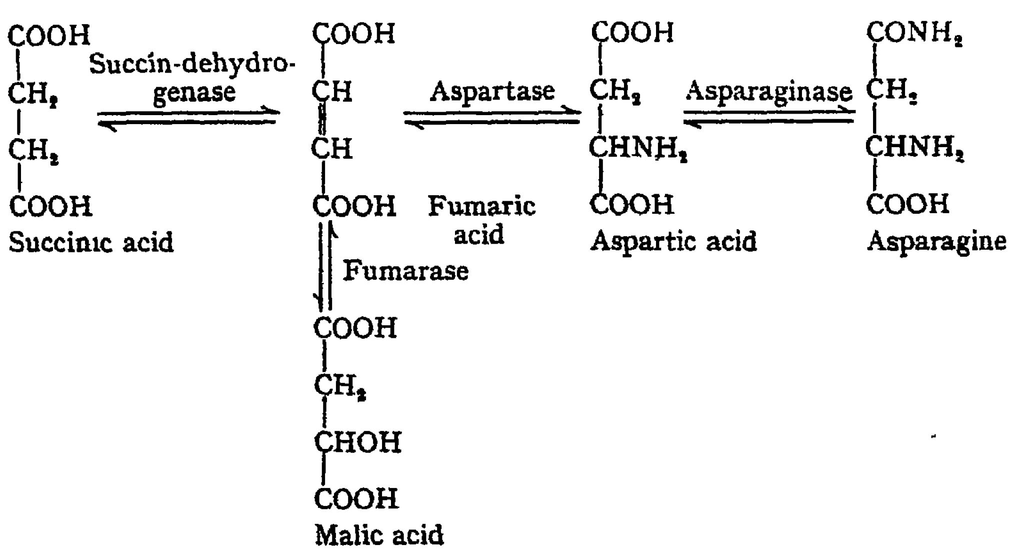
The distinctive role, if any, of glutamine in the plant is still obscure. It is known to be the chief amide present in the Caryophyllaceae, and it frequently occurs together with asparagine, as in starved tobacco and barley leaves, though the two amides seem not to accumulate to their maximum extent at the same time. In 1934 Vickery could still write "glutamine has only rarely figured in the literature", but the publication by Chibnall & Westall (1932) of a method by which the two amides can readily be estimated in the presence of one another has done much to repair this omission. In *Lolium perenne*, grown with a high supply of ammonium nitrogen, Greenhill & Chibnall (1934) found as much as 32 per cent of the water-soluble nitrogen in the form of glutamine, some of which is actually excreted from the leaf as an exudation which deposits the amide as a solid on evaporation. Vickery *et al.* (1934) found that tomato plants whose nitrogen supply was in the form of ammonium sulphate contained very large amounts of glutamine, which could be isolated from extracts to the extent of 63 per cent of the total amide, in spite of its great solubility and consequent difficulty of isolation. The tomato and sugar-beet respond to large supplies of ammonium nitrogen by the formation of glutamine (Vickery *et al.*, 1936), while the tobacco plant forms both asparagine and glutamine. The effect of nitrogen supplied as ammonium salts and as nitrates on the nitrogenous constituents of the tomato has been studied by Clark (1936), who finds that the content of glutamine and asparagine is much higher in the plants supplied with ammonium salts. The plants with nitrates grew better.

It may be mentioned that glutamic acid is the only amino-acid which is found (Weil-Malherbe, 1936) to be oxidized by a number of animal tissues, such as brain, which normally only oxidize carbohydrate. Brain tissue oxidizes glutamic acid to α-hydroxyglutaric acid and ammonia, the latter combining with excess glutamic acid to form glutamine, which disappears without forming ammonia, so that there appears to be some sort of "glutamine cycle" in the tissue. These results, together with the marked instability of glutamine to acid hydrolysis, suggest that it and glutamic acid may be the forms in which nitrogenous compounds are most readily broken down to ammonia and non-nitrogenous products in the plant.

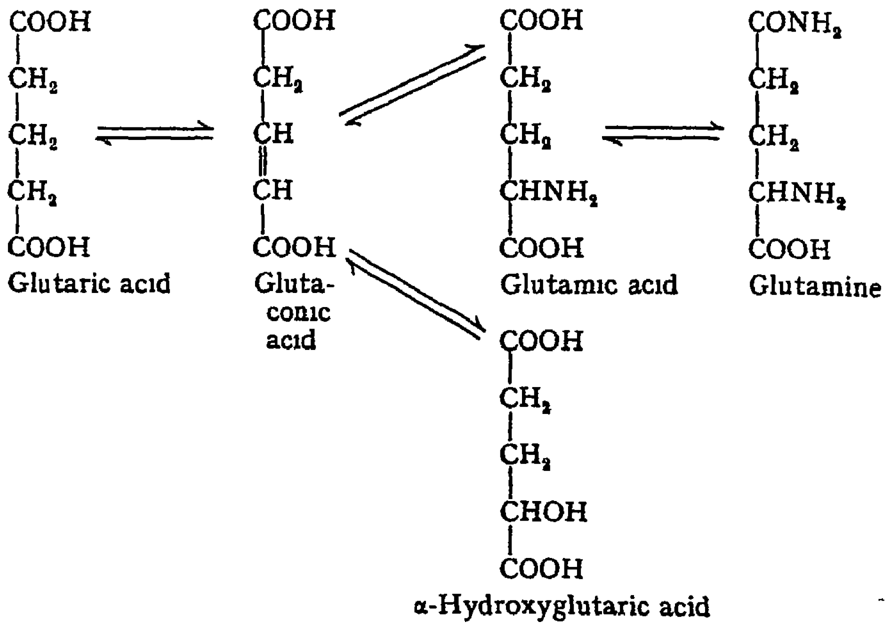
There is little direct evidence as to the reactions by which the amides are formed in the plant. Asparagine is the γ-amide of α-amino succinic acid, and glutamine the corresponding derivative of α-aminoglutaric acid, so that *a priori* a connexion with the dicarboxylic acids

may be expected. Mothes (1933) showed that leaves supplied with ammonium succinate, malate and fumarate by vacuum infiltration formed considerable amounts of amide. It has since been shown, however, by Schwab (1935) that if the sodium salts of these acids are infiltrated there is no subsequent amide formation which indicates that that found by Mothes was due rather to the added ammonium than to the fact that the carbon chains of the acids are related to that of asparagine. This argument is not entirely rigorous, for the absence of ammonia in Schwab's experiments might prevent amide formation even in the presence of suitable non-nitrogenous compounds. Schwab also found that if ammonium salts of organic acids are injected into leaves, the nature of the plant and not the chemical structure of the acid determines whether glutamine or asparagine is formed. It may be noted that Clark (1936) has found that tomato plants grown with nitrates contain much larger amounts of oxalic, malic and citric acids than those grown with ammonium, while the latter have a much higher amide content.

The following schemes for the formation of asparagine (IV *a*) and glutamine (IV *b*) have been suggested by Greenhill & Chibnall (1934). The reactions involved in the asparagine scheme are all known to be catalysed by enzymes. An enzyme aspartase, which catalyses the reaction aspartic acid $\rightleftharpoons$ fumaric acid and is strictly specific, has been found in pea seedlings and red clover by Virtanen & Tarnanen (1932). Asparaginase, which catalyses the splitting of ammonia from asparagine, has been extracted from yeast by Grassmann & Mayr (1933). It is strictly specific, except for the fact that aspartic acid diamide is also hydrolysed, though much more slowly than asparagine. There is not



......(IV *a*)



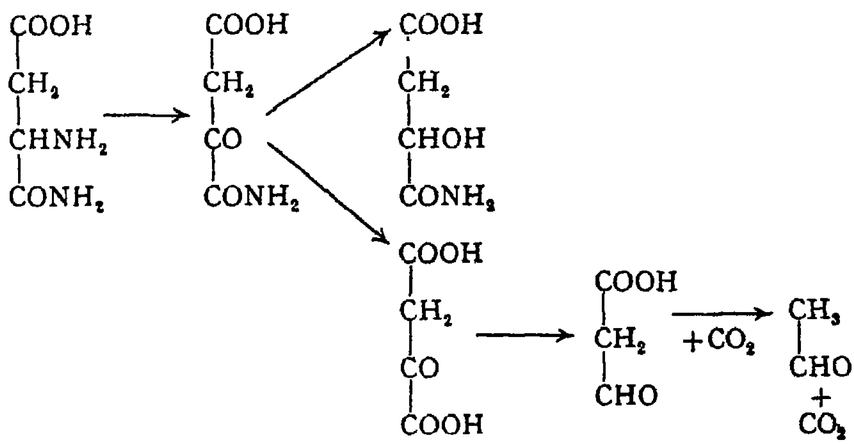
......(IV*b*)

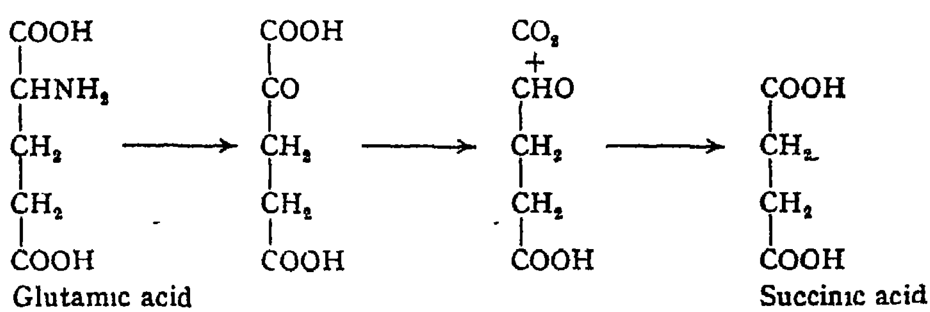
at present as much evidence for a corresponding scheme for glutamine, but glutaric and α-hydroxyglutaric acids have been found in sugar-beet, which is also rich in glutamine. It is possible that if other plant materials come to be investigated as thoroughly as beet residues (a favourite object for investigation on account of their industrial importance and ready availability) these acids will be found to be widely distributed. Krebs (1933) has shown that α-hydroxyglutaric acid is formed in the deamination of glutamic acid by kidney tissue. D. M. Needham (1930) has found that in muscle glutamic acid is a precursor of succinic acid, while a mixture of glutamic and aspartic acids leads to the formation of succinic, fumaric and malic acids. The corresponding synthesis of aspartic acid from fumaric acid and ammonia has been demonstrated by Jacobsohn *et al.* (1935) and Glimm & Nitzsche (1932) have suggested the following scheme (V) to represent the breakdown of aspartic and glutamic acids by yeast. Schwab (1936) has confirmed the specificity of yeast asparaginase, but has found that barley extracts produce ammonia both from asparagine and from glutamine. Jacobsohn & Soares (1936) have found that an aspartase preparation from *B. coli* was highly specific, but catalysed the addition of hydroxylamine and hydrazine to fumaric acid as well as that of ammonia, an oxyaspartic acid and diaminosuccinic acid being formed.

In the animal body glutamine can also be formed from proline and histidine. Weil-Malherbe & Krebs (1935) found that rabbit kidney converts proline to glutamine, and this has been confirmed by Neber

(1936) who found that neither pyrrolidonecarboxylic acid nor α-amino δ-oxyvalerianic acid appears as an intermediate product and concluded that there is a direct oxidative splitting of the proline ring, leading to the formation of glutamic acid, as happens *in vitro* with hydrogen peroxide. The changes in the formation of glutamine from histidine, as formulated (VI) by Edlbacher & Neber (1934), are somewhat similar.

Proline and histidine are present in appreciable quantities in a number of plant proteins. Blagoveshchenski & Schubert (1934) found 14·29 per cent by weight of histidine and 5·26 per cent of proline



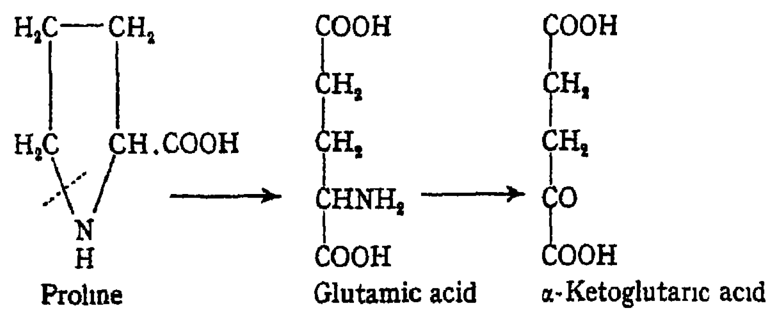


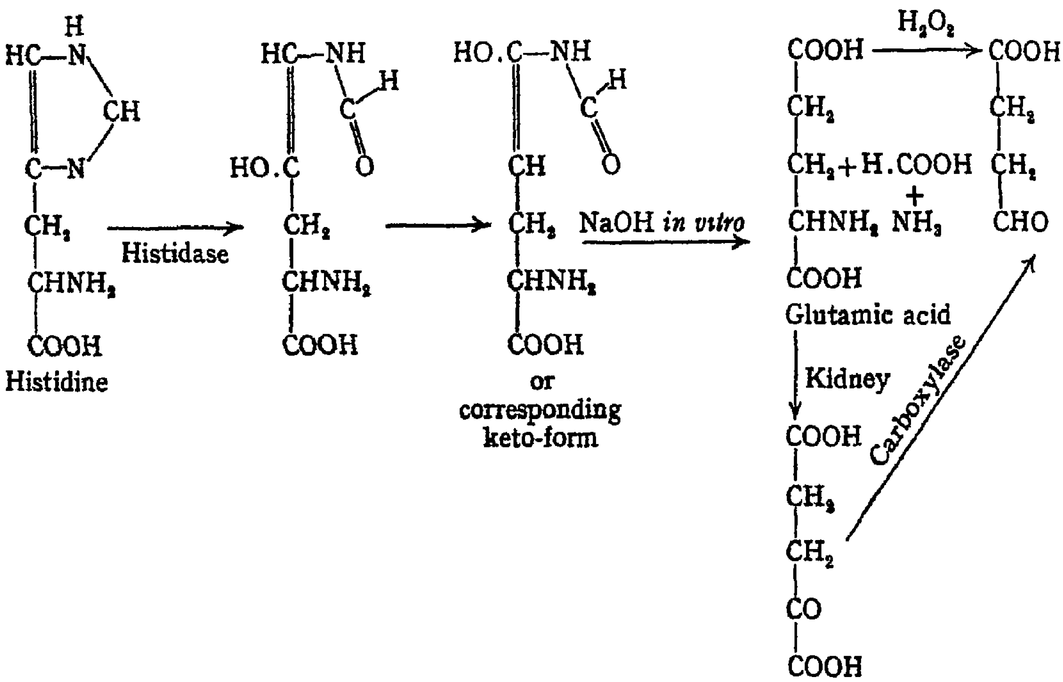
......(V)

in the protein of sunflower seeds, and Miller (1936) has shown that 2 per cent of the nitrogen of the protein extracted from the leaves of *Dactylis glomerata* is accounted for by proline. It is thus possible that these amino-acids may serve as sources of glutamine in the plant also.

In spite of our increased knowledge of the occurrence of the acid amides and of their metabolic relations in the plant it is still not possible to decide finally between the two hypotheses of Schulze and Pryanishnikov. It is clear that the amides do actually act as a means of storing ammonia in a harmless form, if it is present in excess, which can only rarely occur in normal conditions, but it is not therefore certain that they have no other part in plant metabolism. They

arise in leaves deficient in carbohydrates, and Burkhart (1934) has reported similar results with leguminous seedlings. In detached leaves the amides arise from the products of protein breakdown, but in the seedling they might equally well be up-grade products which accumulate because the shortage of carbohydrate prevents their further synthesis to protein. The molecule of asparagine has four carbon atoms and two nitrogen atoms, which gives a value of 1·7 for the



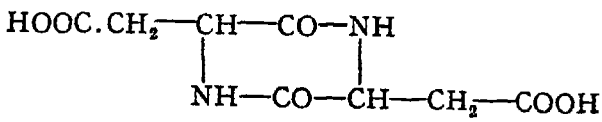


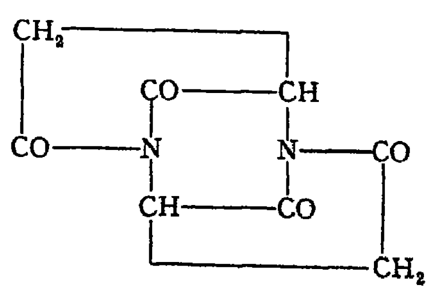
......(VI)

C/N ratio by weight. In the average protein (Karrer, 1933) this ratio is about 3·2 and there are nearly four carbon atoms for each one of nitrogen. It is clear that for the formation of protein from asparagine two carbon atoms must be supplied from other sources, presumably carbohydrate or its breakdown products, for each atom of nitrogen used.

The work of Björkstén (1930) and of Björkstén & Himberg (1930) (see p. 41) shows that amides may take part in protein synthesis

without the formation of free ammonia, though there is still no direct evidence that the carbon chain of the amide is used in the synthesis, as was suggested by E. Schulze. It is clear that our views on this matter must ultimately depend on our knowledge of the structure of the plant proteins. If they are formed by repeated condensations of amino-acids through peptide linkages, as suggested by Emil Fischer, then all the amino-acids formed on the hydrolysis of a given protein are required for its synthesis, and the amides can take part as such only to the extent to which they are found in the protein molecule. As glutamic acid and ammonia, which probably arise from glutamine units in the protein molecule (Damodaran *et al.* 1932), are formed in large amounts in the hydrolysis of most plant proteins, glutamine might take part in protein formation in this way on a comparatively





......(VII)

large scale. If, on the other hand, the protein molecule is built up of complex ring systems, as was also first suggested by Emil Fischer, and has more recently been reaffirmed by Abderhalden, the condensation product of a small number of units, polymerized to the extent found in such other plant products as rubber and cellulose, could give rise on hydrolysis to a large number of different amino-acids. This theory of protein structure, while still unproven, has been rendered more probable by the work reviewed on p. 43. It may be mentioned that Blanchetière (1924) found that aspartic and glutamic acids can form two types of cyclic anhydrides, the first being a normal diketopiperazine derivative, the second a three-ringed compound (VII). It is possible that the flexibility of chemical behaviour which the presence of two carboxyl and one amino groups confers on glutamic and aspartic acids is of importance in the formation of proteins in the plant.

THE NITROGEN METABOLISM OF "ACID" PLANTS

Certain new ideas on the nitrogenous metabolism of the plant have been introduced by Ruhland & Wetzel (1926, 1927, 1929), whose work has a comparative basis, a merit distinctly unusual in plant physiology. They consider the two metabolic groups, of "acid plants" and "amide plants", which, however, have no taxonomic unity. The latter have a cell-sap reaction higher than about *p*H 5, and their metabolism is of the type described by E. Schulze, Pryanishnikov & Mothes in their work on amides. The "acid plants" have more acid cell saps and, according to Ruhland & Wetzel, they react in an entirely different way to the presence of excessive ammonia in the cell. The cell sap of these plants contains large amounts of organic acids, 20 per cent of the dry weight of *Begonia semperflorens* being oxalic acid, with malic and succinic acids also present. Ruhland & Wetzel, following up a suggestion of Kostychev, regard the acids as being produced by the oxidative deamination of amino-acids rather than as by products of carbohydrate breakdown. The ammonia produced in deamination is rendered harmless by the simultaneous production of the acids, so that they perform the same function, the removal of excess ammonia, as the amides in plants with less acid saps.

The theory is plausible qualitatively, but it has been severely criticized on quantitative grounds by Bennet-Clark (1933) who maintains the older view that the organic acids arise in carbohydrate metabolism. Bennet-Clark & Woodruff (1935) found no increase in ammonia or acid in sprouting rhubarb rhizomes, where deamination might be expected to occur, and is indeed stated to occur by Ruhland & Wetzel (1927). The ratio of the ammonia produced in the tissues of the rhubarb to the malic acid was usually about 0·1, while the theory that both arise in amino-acid deamination requires a value of 1. Hulme (1936), in a study of the nitrogen metabolism of the apple fruit, found that, in spite of the acid sap of the variety used (Bramley's Seedling), there was always much more asparagine present than ammonia, and Culpepper & Caldwell (1932) were unable to demonstrate the simultaneous formation of organic acids and ammonia in rhubarb, though Schwarze (1932) states that they increase together in *Oxalis Deppei* exposed to high temperatures in the dark. Pucher *et al.* (1937*a*, *b*) find that the ammonia content of rhubarb is always low and shows no correlation with total acidity or with the concentration of any individual acid.

These objections to the Ruhland-Wetzel theory seem to be well founded, at least for rhubarb, where the alternative theory of Bennet-Clark is more in accordance with the available facts, but they all refer to the theory of the origin of the acids rather than that of their action in the plant. Whatever their origin, they are undoubtedly present in these plants, and if the ammonia concentration should rise in their cells it will be converted to ammonium salts of the acids, so that the "acid" plants have a means of rendering ammonia harmless without amide formation. Garber (1935) found that in "acid" plants treated with gaseous ammonia, ammonium salts of organic acids were formed, and in "amide" plants amides. In both types the supply of ammonia in concentrations lower than 1/7500 led to increased protein formation. Kultscher (1932), working at Leipzig under Ruhland & Wetzel, did urea-feeding experiments with *Begonia hydrocotylifolia*, an "acid" plant, and *Phaseolus vulgaris*, an "amide" plant. Both took up the urea readily and ammonia was formed in their tissues. In the "acid" plant it was stored as the salt of an organic acid, and later synthesized to protein, but the "amide" plant was severely damaged, as its protective mechanism was unable to deal with a sudden large excess of ammonia.

THE ALKALOIDS AND SIMPLER PLANT BASES

Both on account of their chemical complexity and of their striking effects on the animal organism the alkaloids are one of the most interesting special groups of substances produced in plant metabolism. Our knowledge of their chemistry is now considerable, but their metabolic role and their mode of formation in the plant remain in most cases obscure.

Some interesting results have, however, been obtained in the period under review, in spite of the obvious difficulties of this field of research. Weevers (1933) gives a good account of the chemistry of the alkaloids, together with a survey of their distribution throughout the plant kingdom and an account of some work on their physiology. He includes in the term "alkaloid", which is of somewhat vague application, such groups as the betaines and the purine derivatives. Weevers's main conclusions are given in the following quotation: "A detailed account of those alkaloids which are well known chemically shows that they could all be formed, in more or less complicated ways, by biochemically possible reactions from the products of protein breakdown. The closer the connexion of an alkaloid is with these protein

breakdown products, so that it can arise from them by simple chemical processes such as internal anhydride formation, methylation or decarboxylation, the more widely is it distributed through the plant kingdom." This generalization is illustrated by the occurrence of trigonellin, a simple betaine, in twenty-eight families from eleven orders; of caffeine, a purine derivative, in six families from five orders; and of hordenin, which can be derived from tyrosine by decarboxylation and methylation, in the three widely separated families Gramineae, Loranthaceae and Cactaceae. The more complicated alkaloids are mostly confined to small groups of genera or of species, though berberine, which has a complex structure, has been reported from six families.

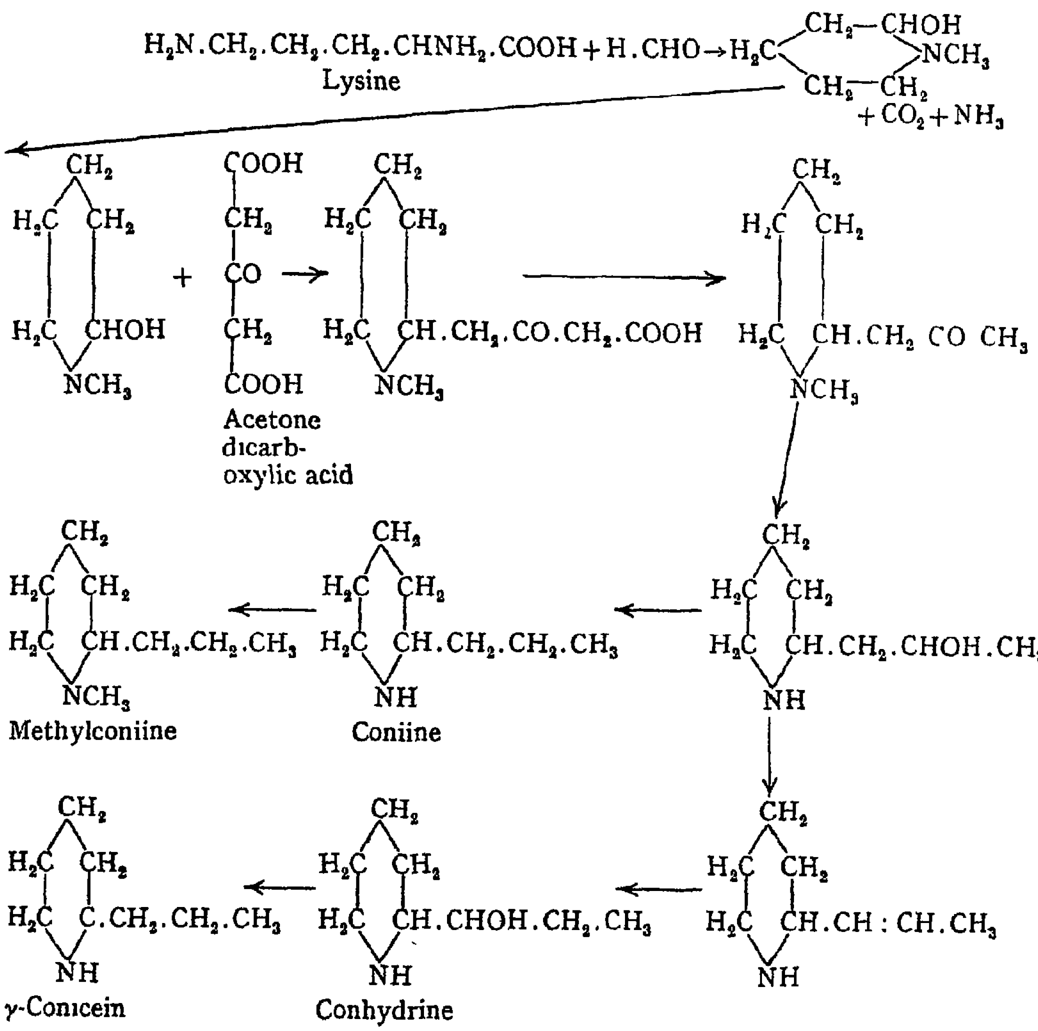
McNair (1934) has shown, in a statistical survey of the alkaloids, that those which are confined to one family have a higher average molecular weight than those which are more widely distributed. He also found a significant correlation between the position of a family in a standard classification and the complexity of its alkaloids, the more advanced families having the more complex alkaloids.

The idea of the formation of the alkaloids from the products of protein breakdown has also been stressed by R. Robinson (1917) who has put forward the following scheme (VIII) for the formation of the piperidine alkaloids in the plant and similar schemes for the formation of other groups. These reactions are plausible on chemical grounds and only involve compounds likely to be present, but there is still no direct evidence that they occur in the plant.

The general idea of the formation of alkaloids from protein breakdown products is supported by the observation of Weevers (1933) that in etiolated *Ricinus* seedlings, where considerable protein breakdown occurs, the ricinin content increases until it accounts for 6·4 per cent of the total nitrogen of the seedling, compared with 0·4 per cent in the seed, and by the similar results of Sukhorukov & Borodulina (1932) working with *Datura Stramonium* and with *D. meteloides*. Cromwell (1933) showed, by ringing experiments on *Berberis Darwinii*, that berberine arises indirectly from protein breakdown products. Chaze, in a series of papers (1927, 1929, 1931), describes his histochemical studies on the formation of nicotine in the tobacco plant. He finds that in the seedlings nicotine is present in the vacuoles of all active cells, but that in mature plants it tends to migrate to the peripheral parts.

The alkaloids have been widely regarded as waste products of metabolism, but while this is probably true for the more complex

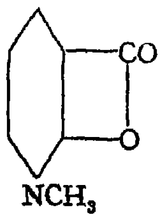
members of the group, there is evidence that the simpler ones can be utilized by the plants which form them. Cromwell (1933) found that in *B. Darwinii* berberine was formed during the period of active growth and accumulated in the cortex and bark. It was not broken down in the winter, so that the total alkaloid content increased from year to year. There is some evidence that nicotine, which has a much

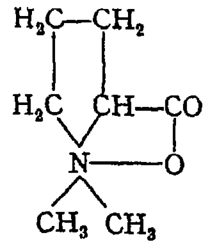


......(VIII)

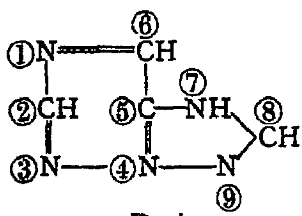
simpler structure than berberine, can be utilized by the plant as a source of nitrogen. Vickery *et al.* (1933) find that in detached tobacco leaves there is a gradual loss of nicotine following an initial synthesis, and similar observations have been made by Smirnov & Izvoshikov (1930). Smirnov *et al.* (1928) find a steady increase in the nicotine content of tobacco leaves during development, except for a break at the time of flowering, which might be due either to an actual utilization of nicotine or to a diversion of nitrogen from nicotine formation

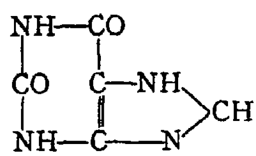
to flower building. According to Ilyin (1934) the nicotine content of ripening seeds decreases considerably after an early maximum, which is in agreement with the general decrease in the simpler nitrogenous compounds and increase in protein in ripening seeds, and with the


Trigonelline

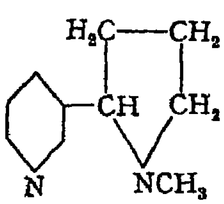

Stachydrine

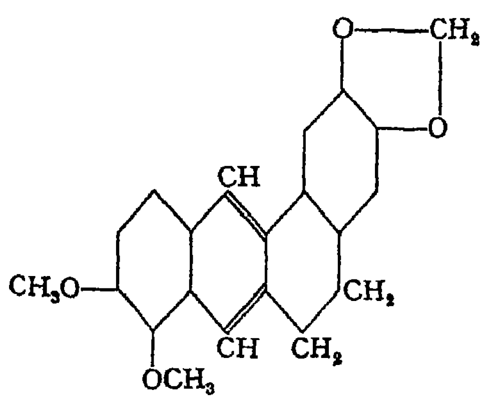
Betaines

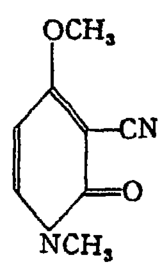

Purine


Xanthine

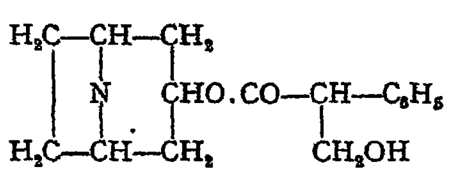
Purines


Nicotine


Berberine


Ricinin

Alkaloids


Atropine, one of the *Datura* alkaloids

......(IX)

very low alkaloid content of the seeds of many alkaloid plants. The fate of the lost nicotine was not determined by Ilyin, but the work of Vickery & Pucher (1929) shows that it is not likely that any important amount was lost by volatilization.

Chaze (1931) has shown that measurable amounts of nicotine are lost by volatilization from tobacco leaves, but the amounts involved are very small, and according to Vickery & Pucher (1929) the reaction of the cell sap is about *p*H 5·5 and at an acidity greater than *p*H 6 not more than 1 per cent of the nicotine is present as the free base, i.e. in a volatile form. Chaze (1931) also found that some alkaloid was lost by exudation from the leaf, though it does not appear that the loss in this way was sufficient to account for the decrease observed in detached leaves.

There is much more convincing evidence that the relatively simple xanthine derivatives normally act as nitrogen reserves in the plant. Weevers (1930) has investigated the metabolism of the xanthine bases in species of *Thea, Coffea, Kola, Ilex, Paullinia* and *Theobroma* and finds that in old leaves they are converted to other substances and translocated to other parts of the plant. He concludes. "The xanthine bases thus arise from the products of protein breakdown, and form a convenient type of nitrogen store; in the seed they are to be regarded as reserve substances."

Most of the purine bases found in plants are derived from xanthine (2:6-dioxypurine); for example theobromine (3:7-dimethylxanthine), theophylline (1:3-dimethylxanthine), caffeine (1:3:7-trimethylxanthine) and 3-methylxanthine all occur in tea leaves together with xanthine itself and adenine (6-aminopurine). Hypoxanthine (6-oxypurine) and guanine (2-amino-6-oxypurine) also occur in the potato.

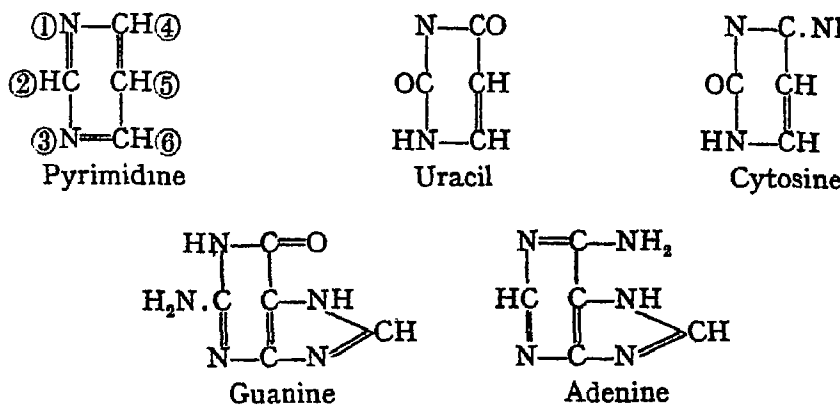
The purines are of particular interest through their relation to the nucleic acids. A typical nucleic acid may be formulated thus:

```
Phosphoric acid—pentose—guanine
                   |
Phosphoric acid—pentose—adenine
                   |
Phosphoric acid—pentose—cytosine
                   |
Phosphoric acid—pentose—uracil
```

As well as the purine derivatives guanine and adenine, the pyrimidine derivatives cytosine (2-oxy-6-aminopyrimidine), thymine (2:6-dioxy-5-methylpyrimidine), uracil (2:6-dioxypyrimidine) and 5-methylcytosine are found in nucleic acids. The purine or pyrimidine is joined to a pentose, or as in a nucleoside isolated from yeast by Suzuki *et al.* (1924) and Levene & Sobotka (1925) to a thiopentose, to form a nucleoside, which combined with phosphoric acid forms a nucleotide. Four nucleotides combined as shown above form a

nucleic acid, and chromatin, the material of which the chromosomes are formed, is considered to consist mainly of nucleic acids combined with protamines, a simple type of protein. The co-enzyme of yeast fermentation is also an adenine nucleotide (Myrbäck, 1933).

Purucker (1932), who has studied the allantoin metabolism of borage seedlings, suggests that in the plant there may be two types of nitrogen metabolism (XI). Purucker found that the production of allantoin was associated with loss of purines and decided as a result of feeding experiments that uric acid was the immediate precursor. He was not able to demonstrate its presence in his plants, but it has



Pyrimidine and purine derivatives found in nucleic acids.

......(X)

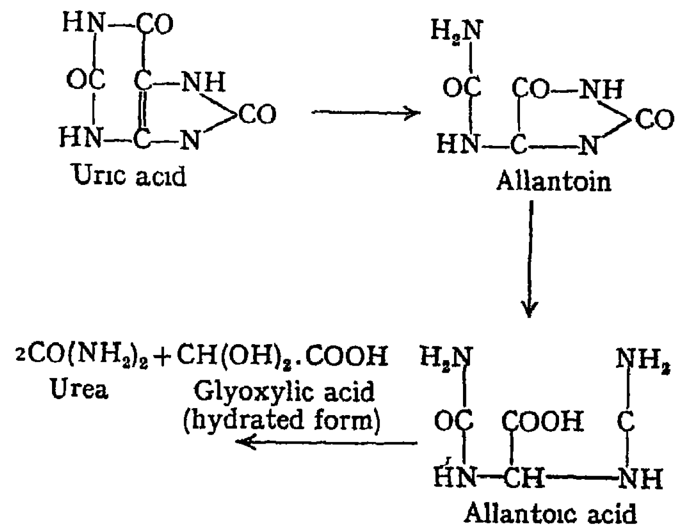
Nuclein	Protein
↓	↓
Purines	Amino-acids → Amides
↓	↓
? Urea	Ammonia
↓	
Allantoin	
Nucleus	Protoplasm

......(XI)

since been found in *Melilotus* by Fosse *et al.* (1932) and in *Sorghum halepense* and a number of legumes by Michlin & Ivanov (1936). Sumi (1928) also found it in the spores of *Aspergillus oryzae*. In the vertebrates uric acid is formed from xanthine by xanthine oxidase, but Michlin & Ivanov (1936) were unable to demonstrate the presence of this enzyme in the plants in which they found the acid. It is thus of interest to note that in the snail an alternative method of uric acid formation has been found by Baldwin (1935). Here it is formed from urea and tartronic acid (HOOC.CHOH.COOH) and it it possible that other three-carbon acids can replace tartronic acid. Fosse and his co-workers (1929*a*, *b*, 1930, 1932) believe that the sequence uric

acid → allantoin → allantoic acid → glyoxylic acid + urea occurs in the plant.

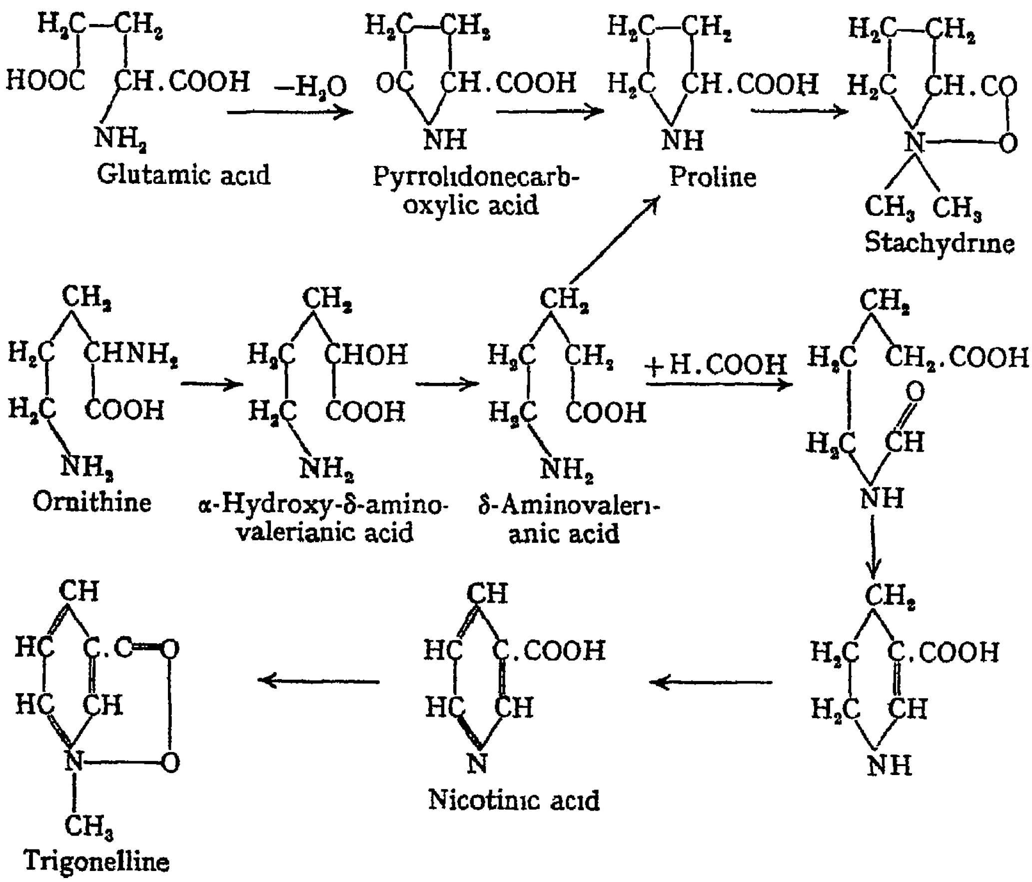
Some interesting work on the mode of formation of the betaines has been done by Klein & Linser (1932, 1933*a*, *b*), who supplied plants which form these substances with various compounds which were suspected on chemical grounds of being concerned in their synthesis. These were sometimes introduced into the leaves by vacuum infiltration, but it was found that injection into hollow stems was more effective. In *Trigonella foenum graecum* and *Dahlia variabilis* increased synthesis of trigonelline followed injection of ornithine, glutamic acid and especially of proline plus urotropine (hexamethylenetetramine), a condensation product of formaldehyde with



......(XII)

ammonia, which readily liberates formaldehyde, probably the chief methylating agent in the plant. Proline alone gave rise only to a slight betaine synthesis. A number of other amino-acids, including aspartic acid, leucine, citrulline and arginine, had no effect. Proline was also used in stachydrine formation by *Stachys palustris*, *S. recta* and *Galeopsis ochroleuca*. The formation of stachydrine from proline is not surprising, as they are chemically related, but in the formation of trigonelline there is a transition from a five- to a six-membered ring. Klein & Linser (1933*c*) also reported that proline, ornithine and glutamic acid increased nicotine synthesis in *Nicotiana tabacum*, but this work has been criticized by Gorter (1936) who was unable to confirm their results. In Klein & Linser's experiments tobacco plants were grown in solutions of the amino-acids, and controls in culture solution. Gorter found that at the end of 14 days there was indeed

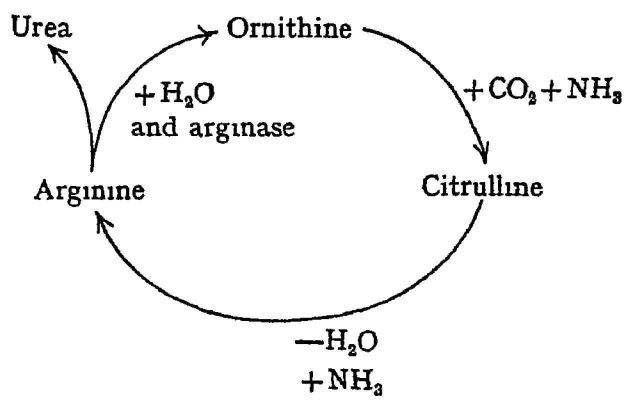
more nicotine in the treated plants than in the controls, but that in both there was less than at the start of the experiment. Klein & Linser suggest the following scheme (XIII) for the formation of the betaines in the plant. Klein and his co-workers have also studied the metabolic relations of arginine, urea and choline in plants. Arginine is an important constituent of most plant proteins, accounting for 13·6 per cent of the nitrogen in the protein extracted by Miller (1935) from *Dactylis glomerata* leaves, and for between 10 and 15 per cent of the



......(XIII)

nitrogen in a number of seed proteins (Onslow, 1931). It is particularly prominent in the nitrogen metabolism of the conifers, where it occurs in large amounts in the seed proteins and in the young seedlings. Klein & Tauböck (1932*a*, *b*) find, in agreement with Mothes (1929), that free arginine is rapidly produced in the young conifer seedling, where it accumulates and does not seem to be readily utilized. Arginine synthesis only occurs on a large scale in the conifers during the ripening of the seeds. Arginine accumulates more in etiolated than in green seedlings, which may be due to decreased utilization in carbohydrate deficiency, but is more probably to be ascribed to

increased protein breakdown. Mothes (1929) suggests that arginine is used in protein synthesis without being further broken down, but Klein & Taubock (1932*a*, *b*) find that it can also be broken down with the formation of urea, both in conifer seedlings and in other plants supplied artificially with arginine. In maize, which is normally urea-free, arginine feeding led to its formation, but it did not accumulate to as great an extent as in *Phaseolus vulgaris*, which does contain urea. Kiesel (1922*a*, *b*) has detected arginase, which hydrolyses arginine to ornithine and urea, in a number of plants, and has found ornithine in *Vicia sativa*. The connexion of arginine with urea production is particularly interesting in view of recent work on urea formation in the animal. Solovyev & Mardashev (1932) showed that the urea which appears in the autolysis of liver tissue is formed by the breakdown of arginine, and Krebs & Henseleit (1932), working with



$NH_2.C:NH.NH.CH_2.CH_2.CH_2.CHNH_2.COOH$
Arginine
$NH_2.CH_2.CH_2.CH_2.CHNH_2.COOH$
Ornithine
$NH_2.CO.NH.CH_2.CH_2.CH_2.CHNH_2.COOH$
Citrulline

......(XIV)

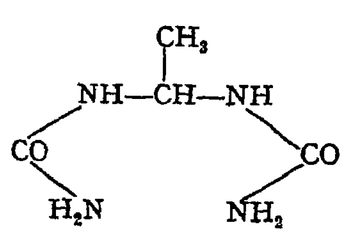
liver slices *in vitro*, concluded that urea is formed as in the following scheme, and not directly from ammonia and carbon dioxide, as had previously been supposed. The work has been extended by Manderscheid (1933), who used frog and tortoise material and concluded that the mechanism was general among the vertebrates. London *et al.* (1934) state, however, that they have been unable to confirm Krebs's results in work on the intact dog and this has been repeated, with further evidence, by London & Alexandry (1937). There has resulted a polemic about the interpretation of London's results and the relative value of work *in vitro* and *in vivo* (Krebs, 1934*a*; London,

1934). The latter seems safer at first sight, but the results are complicated by diffusion and other interfering effects. In any case Krebs's results seem to be well founded for liver tissue *in vitro*, and in view of the work of Kiesel (1922*a*, *b*) they may well have an analogy in the plant. The amino-acid citrulline was isolated by Wada (1930, 1933) first from *Citrullus* and later from the products of tryptic digestion of casein.

Urea is not found in large quantities in plant tissues, with one striking exception provided by the fruit-bodies of certain higher fungi (Ivanov, 1923*a*, *b*, 1927). In *Lycoperdon gemmatum* and *Bovista nigrescens* over 30 per cent of the total nitrogen of mature fruit-bodies is in the form of urea, and those of *Psalliota campestris* also have a high urea content. The explanation of this remarkable metabolic peculiarity is not known.

A good summary of our knowledge of urea metabolism in plants and a theoretical discussion are given by Kiesel (1927). The metabolic status of urea is not yet settled, and it is certainly formed in more than one way, as we have seen that it may arise as a breakdown product of arginine (Klein & Taubock, 1932*a*, *b*) and as an end-product of purine metabolism (Fosse, *loc. cit.*). Klein & Tauböck, confirming pre-war work by Fosse, have shown that urea is widely distributed, urea plants being particularly common in the Rosales and Terebinthales.

Urea appears during a prolonged period in leguminous seedlings, but only for a very short time in the cereals. This period is not the same for all the plants of a batch or for all the organs of an individual. Much of the urea of the higher plants is in a form not attacked by urease, probably in aldehyde compounds such as

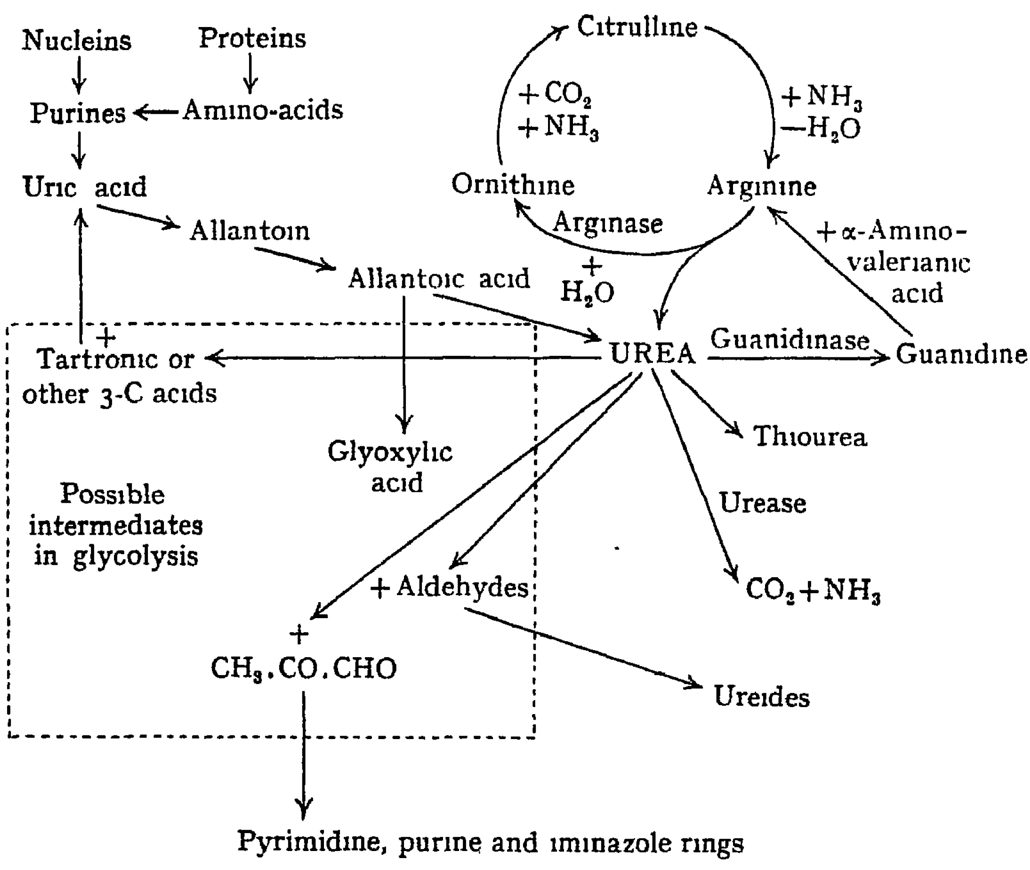


Acetaldehyde ureide

......(XV)

Thiourea has been detected in *Laburnum anagyroides*. Urea and methylglyoxal could, as pointed out by M. E. Robinson (1929), give pyrimidine, purine and iminazole rings by simple condensations, but these suggestions still lack experimental support. The wide distribution of urease, which breaks urea down to ammonia and carbon dioxide,

might be thought to indicate that urea is unlikely to be used in synthesis, but the ureide compounds found by Klein & Tauböck (1932*a*, *b*) remove this difficulty. The enzyme guanidinase, which replaces the oxygen of urea by the imino group, forming guanidine, has been found in *Aspergillus niger* by Ivanov & Ivetisova (1931). Guanidine combines with α-aminovalerianic acid to form arginine. The possible biochemical relations of urea may be summarized in the following diagram:



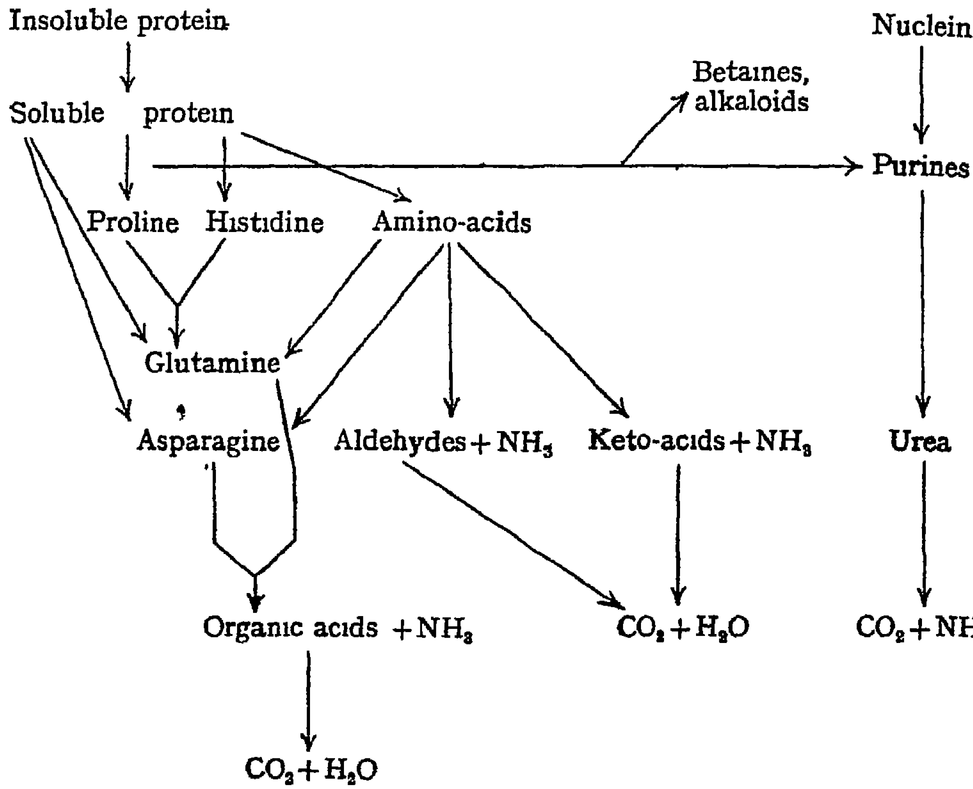
......(XVI)

Choline is best known as a constituent of lecithin, but Klein & Linser (1933*c*) find that in seedlings and mature plants most of the choline present is free, not combined as lecithin. They do not come to any conclusion as to its metabolic function, but Klein & Steiner (1928) suggested that the trimethylamine found in many plants may be formed from choline according to the equation on p. 48. The similarity of structure between choline and muscarine, the poisonous principle of *Amanita muscaria*, may be noted.

Choline	Muscarine
$OH.CH_2.CH_2.N(CH_3)_3.OH$	$(OH)_2.CH.CH_2.N(CH_3)_3.OH$

CONCLUSION

The stages in the formation of protein are still obscure, but the possible sequences in the nitrogen catabolism of the plant may be summarized in the following diagram:



......(XVII)

It is a pleasure to express my gratitude to Dr W. O. James for the interest he has taken in the preparation of this paper, and for his stimulating criticism and advice, and also to Dr E. W. Yemm, with whom I have discussed a number of the problems considered here. I am also indebted to the Department of Scientific and Industrial Research for a maintenance grant for the years 1934–6, and to Trinity College, Oxford, for the renewal of an entrance scholarship.

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THE SECONDARY THICKENING OF *FUCUS*

By WINIFRED PENNINGTON
Department of Botany, University of Reading

(With 6 figures in the text)

Introduction

Although *Fucus* is so frequently studied, its secondary thickening has never been fully investigated. The technique of maceration, by which it can be most easily made out, is now little used with such material. The most important work on the general structure of *Fucus* was published by Reinke in 1876. His paper deals with the vegetative anatomy of this and several other seaweeds, and includes an accurate but incomplete account of the origin and development of the secondary thickening. A later paper by Oltmanns (1889) deals in detail with the segmentation of the apical cell and the early stages in the origin of the primary thallus, but apparently nothing has been added to Reinke's account of the secondary thickening. The account in the recent edition of Oltmanns' *Morphologie und Biologie der Algen*, **2**, is based on Reinke's conclusions.

For his investigation, Reinke used hand sections and material macerated in concentrated hydrochloric acid, and showed that the change from the slender midrib of the young thallus into the thickened stipe of the older plant results from two distinct processes, these being:

(1) The addition of thick-walled hyphae, which arise as filamentous outgrowths from cortical cells and penetrate between the primary cells of the medulla.

(2) Division by tangential walls of cells in the inner cortex, producing in the stipe a tissue of regular cells, similar in appearance to the periderm of woody phanerogams.

This paper is concerned more especially with the first process.

Technique

Fresh and preserved material of the three common species of *Fucus*—*F. spiralis*, *F. serratus* and *F. vesiculosus*—was used for hand sections, which were supplemented by material prepared in one of several ways. Reinke's method of macerating the thallus in con-

centrated hydrochloric acid was tried. Other pieces were prepared by heating the fresh thallus for a few minutes in an autoclave at 30 lb. pressure. (For this convenient method I am indebted to Prof. McLean Thompson.) The most satisfactory material, however, was some very old laboratory material of *F. spiralis*, in which the jelly cementing the cells together had disappeared, though the cell walls were unaltered. Though unfortunately the full history of this material is unknown, it had probably been in dilute formalin for many years.

DESCRIPTION

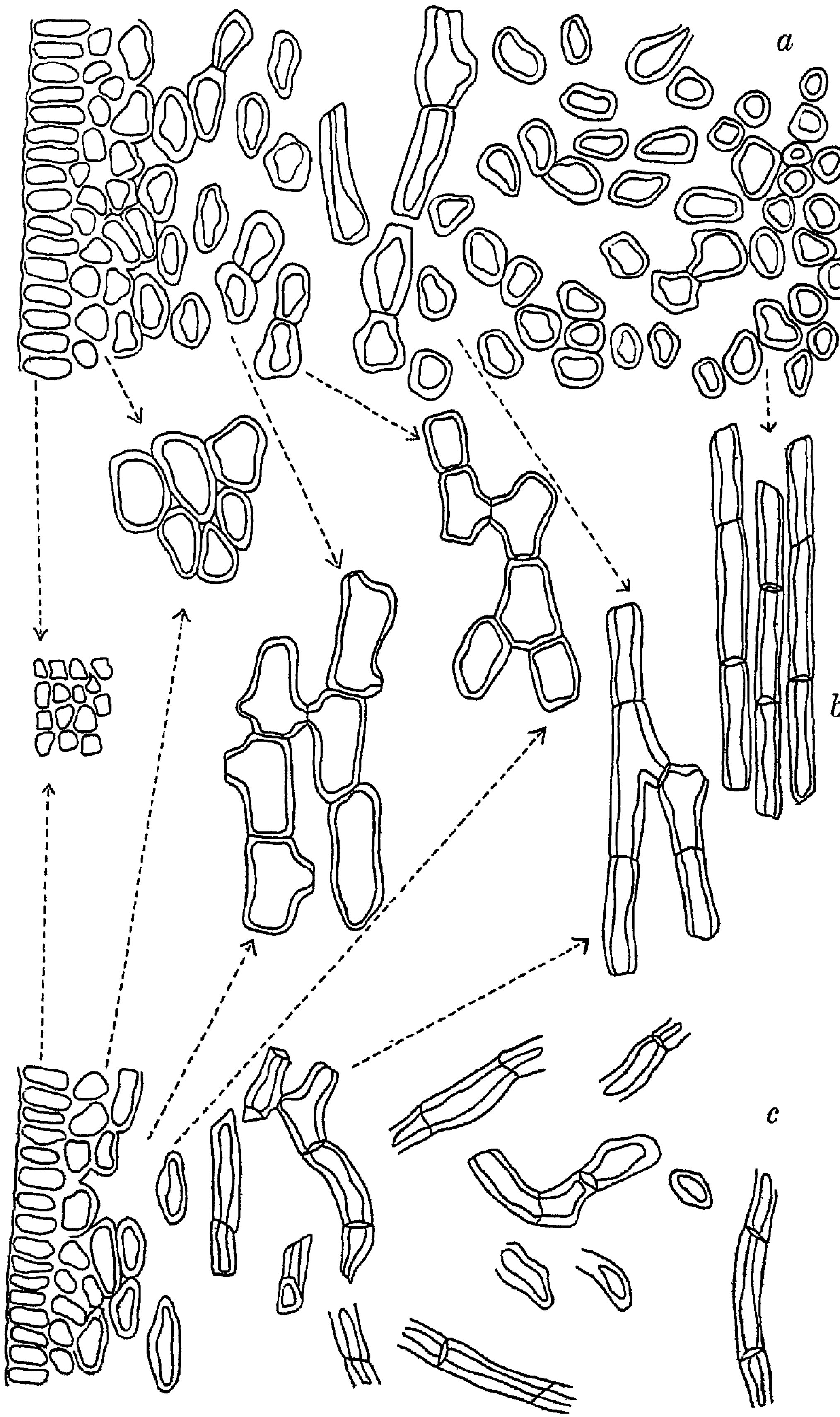
The three species of *Fucus* examined were found to agree closely in structure and the details of their secondary thickening, and in general only *F. spiralis*, the species in most cases figured, need be referred to directly.

There are two phases in the development of the thallus. The primary thallus is produced by rapid subdivision of the products of the apical cell, a process which is completed within about half a centimetre of the tip. Afterwards, there is the slow alteration of the primary thallus into the stipe, by the process of secondary thickening. In the mature plant these two processes are clearly defined, but they tend to overlap in the small frond of the juvenile plant, which ultimately gives rise to the holdfast and the thick base of the stipe. It will be convenient to consider the juvenile plant afterwards.

By the time the plant has reached 6 cm. in height, it is past the juvenile stage, and its young branches show exactly the same primary structure as the young branches of an older plant, with which they also appear to correspond exactly in the stages of their secondary thickening. In these plants, a series of sections working back from the tip of a branch gives the same picture of secondary thickening as a series at the same level from progressively older plants. With plants of less than 6 cm., however, a series of sections at the same distance from the base of progressively older plants is necessary, to show secondary thickening.

Primary structure

The primary structure of the thallus is illustrated in Fig. 1. A comparison of the cells of each tissue in transverse and tangential longitudinal section makes their form clear, and renders a detailed description unnecessary. In the tissues of the wings, i.e. the expanded lamina on either side of the midrib, three layers can be distinguished,



Text-fig. 1. Primary structure of *F. spiralis*, ×250. *a*, transverse section, midrib 5 mm. from tip; *b*, primary cells in tangential longitudinal section; *c*, transverse section, wing 5 mm. from tip.

namely, the outer or palisade layer, the cortex, and the medulla. Plastids are very numerous in the palisade layer, less numerous in the cortex, and usually absent in the medulla. The cortex passes gradually into the medulla with no definite boundary, and the area intermediate between them will henceforward be referred to as the "transition region".

All the cells contain protoplasm, and have rather thick walls, except where they meet another cell. In the inner cortex and medulla, the cells are separated by a clear jelly formed from the outer layers of their walls. The cells of the medulla form longitudinal strings which branch and anastomose with each other, and the cells of the transition region form a closer, irregular network, while the cortex tissue is more like ordinary parenchyma, the cells becoming smaller and more compact as the outer layer is approached.

The midrib differs from the wings in that it contains more cells, and in the medulla especially the tissue is more compact, with less jelly between the cells. The medullary cells run longitudinally and parallel in the midrib, while in the wings they wander freely with a general drift obliquely downwards towards the midrib.

Secondary changes

On passing to older parts of the thallus, the obvious external changes are, first, some increase in the width of the wings, due to enlargement of the cells, and then the disappearance of the wings due to the death and destruction of their cells, and secondly, a steady increase in the thickness of the midrib and the stipe into which it passes, which continues for as long as the plant lives.

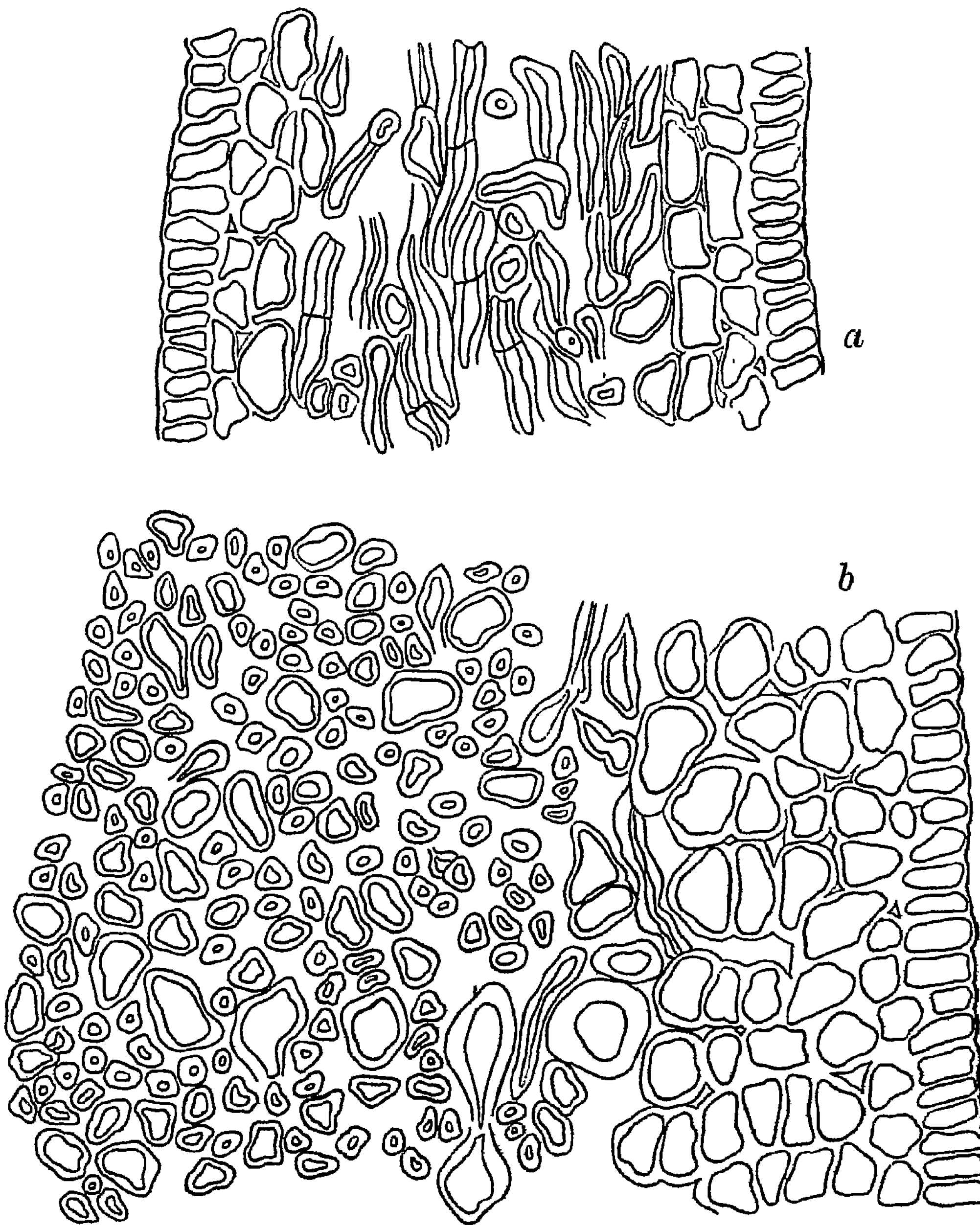
The increase in thickness of the midrib is effected in three ways:

(1) By the considerable enlargement of its cells, especially those of the medulla, which grow to nearly twice their original internal diameter.

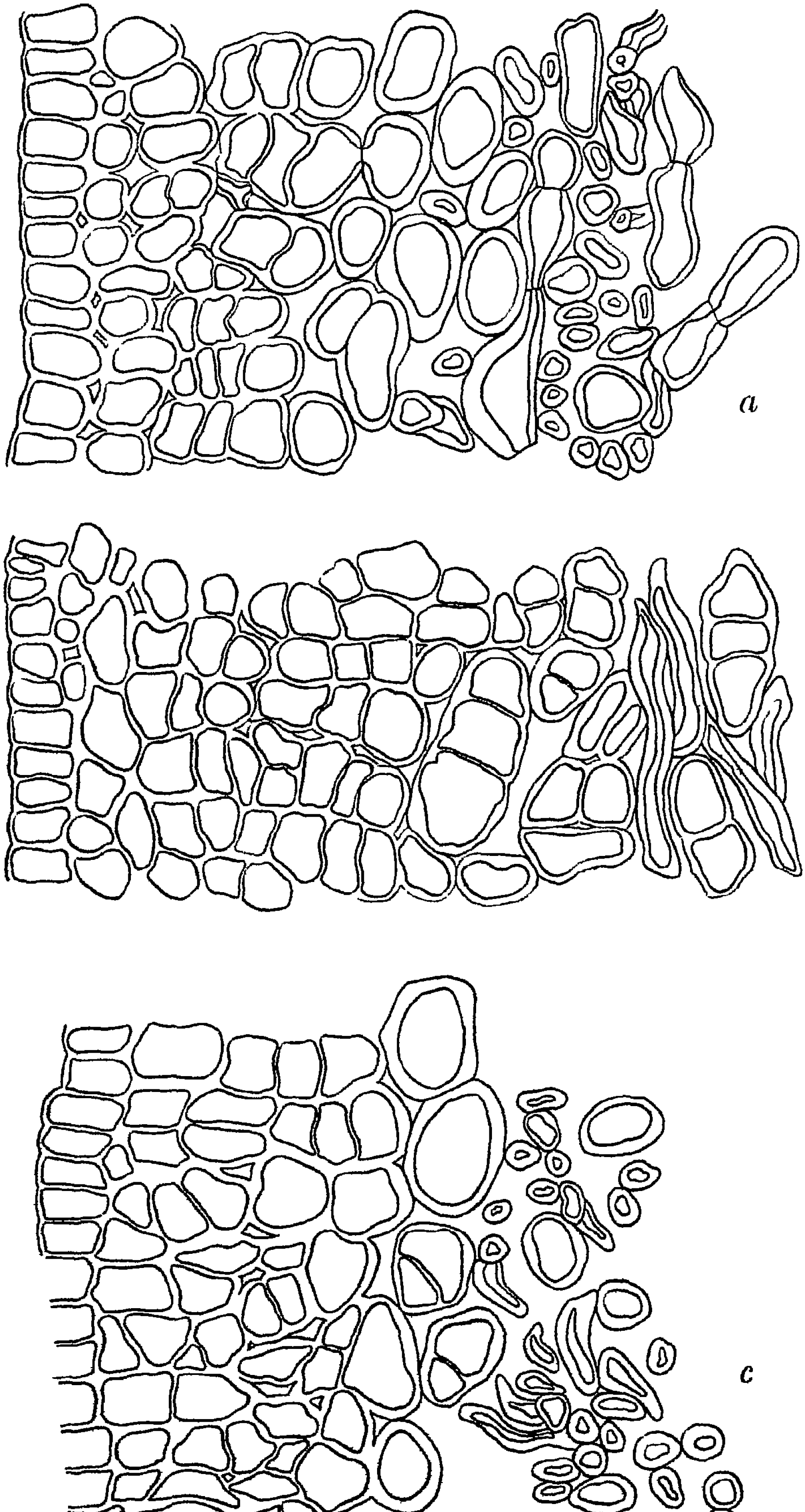
(2) By the addition of new parenchyma to the cortex. Cortical cells divide repeatedly by tangential walls, and form a periderm-like tissue of radially arranged cells, usually called the secondary cortex (Figs. 2*b* and 3). The three species examined differ slightly in the details of this process.

In *F. spiralis* cell division usually occurs in a single irregular layer of cells towards the inner margin of the cortex (Fig. 2*b*).

In *F. serratus* and *F. vesiculosus*, cell division tends to occur nearer the outer boundary of the cortex, and no definite meristematic layer



Text-fig. 2. *F. spiralis*, transverse section 15 cm. below apex of 25 cm plant. ×250. *a*, wing; *b*, midrib.



Text-fig. 3. Outer tissues of older midrib. *a*, *F. vesiculosus*, transverse section, older midrib, ×250; *b*, *F. serratus*, longitudinal section, older midrib ×300; *c*, *F. serratus*, transverse section, older midrib, ×300.

is formed, division occurring simultaneously in at least two layers of cells (Fig. 3). The result of this is that, in the older parts, the radial rows of cells appear to dovetail in with each other. The cells giving rise to this new tissue are cubical or nearly so, which suggests that their formation has involved transverse division of the somewhat elongated cells of the inner cortex (Fig. 3*b*).

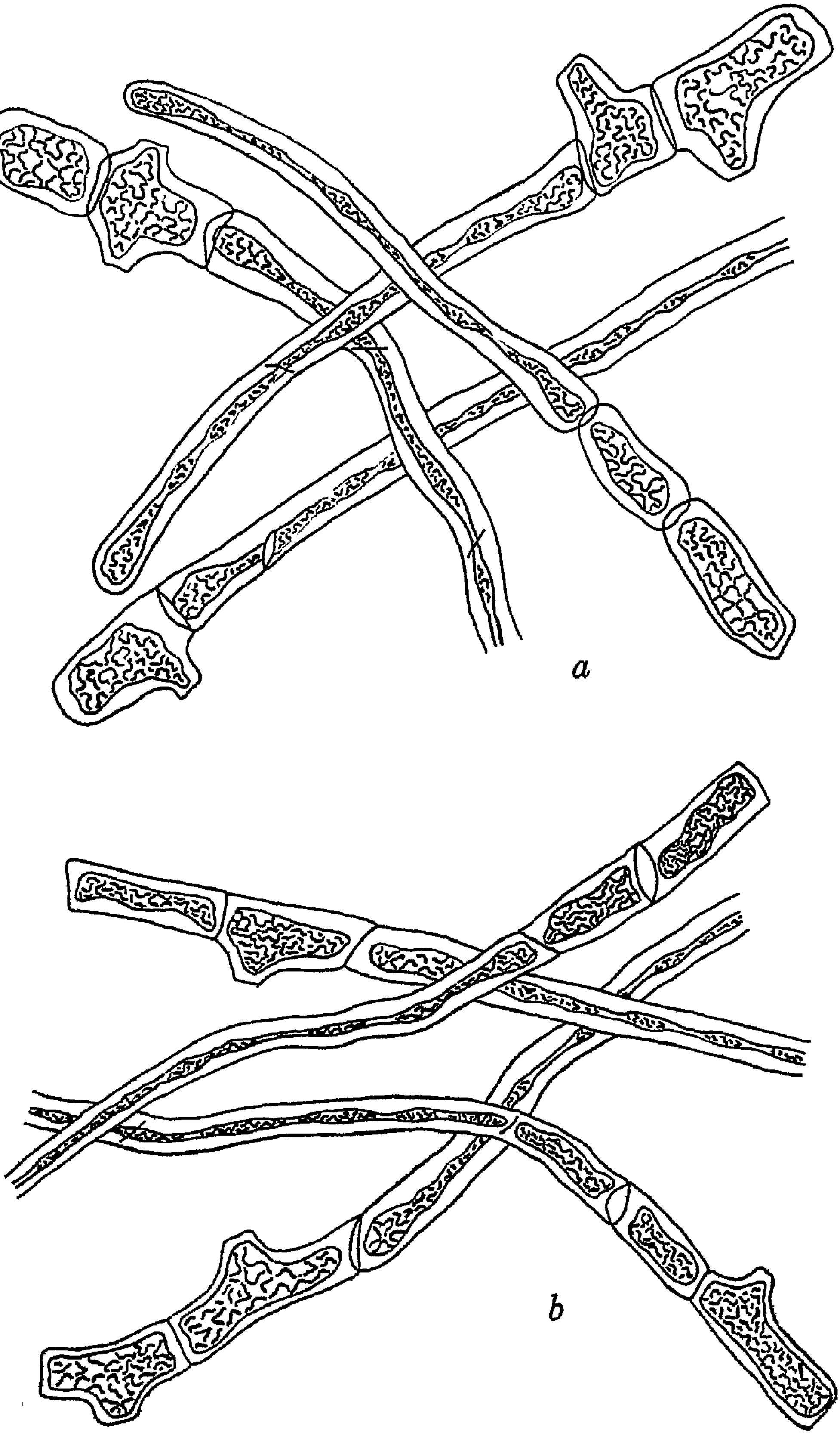
The formation of the secondary cortex begins between 10 and 15 cm. below the apex, just above the region where the wings are disappearing, and it is broadest some distance above the holdfast. Towards the base of the stipe, and in the holdfast itself, there is very little of this tissue.

(3) By the addition of descending "fibres" or "hyphae". These hyphae are filaments of cells, more slender than the medullary filaments, but with thicker walls, and very narrow cavities containing protoplasm. The cells of the hyphae are longer than those of the medulla, and have peculiar end walls which terminate in the mucilaginous substance of the hypha (Fig. 4*a* and *b*). Reinke describes the hyphae as being septate, but does not feature the cross walls in his figures. The hyphae can be differentiated by the method of staining with iodine, followed by a drop of 70 per cent sulphuric acid (Church, 1920). This gives to the walls of hyphae a blue colour, fading in 30 sec., but does not stain the walls of the medullary cells.

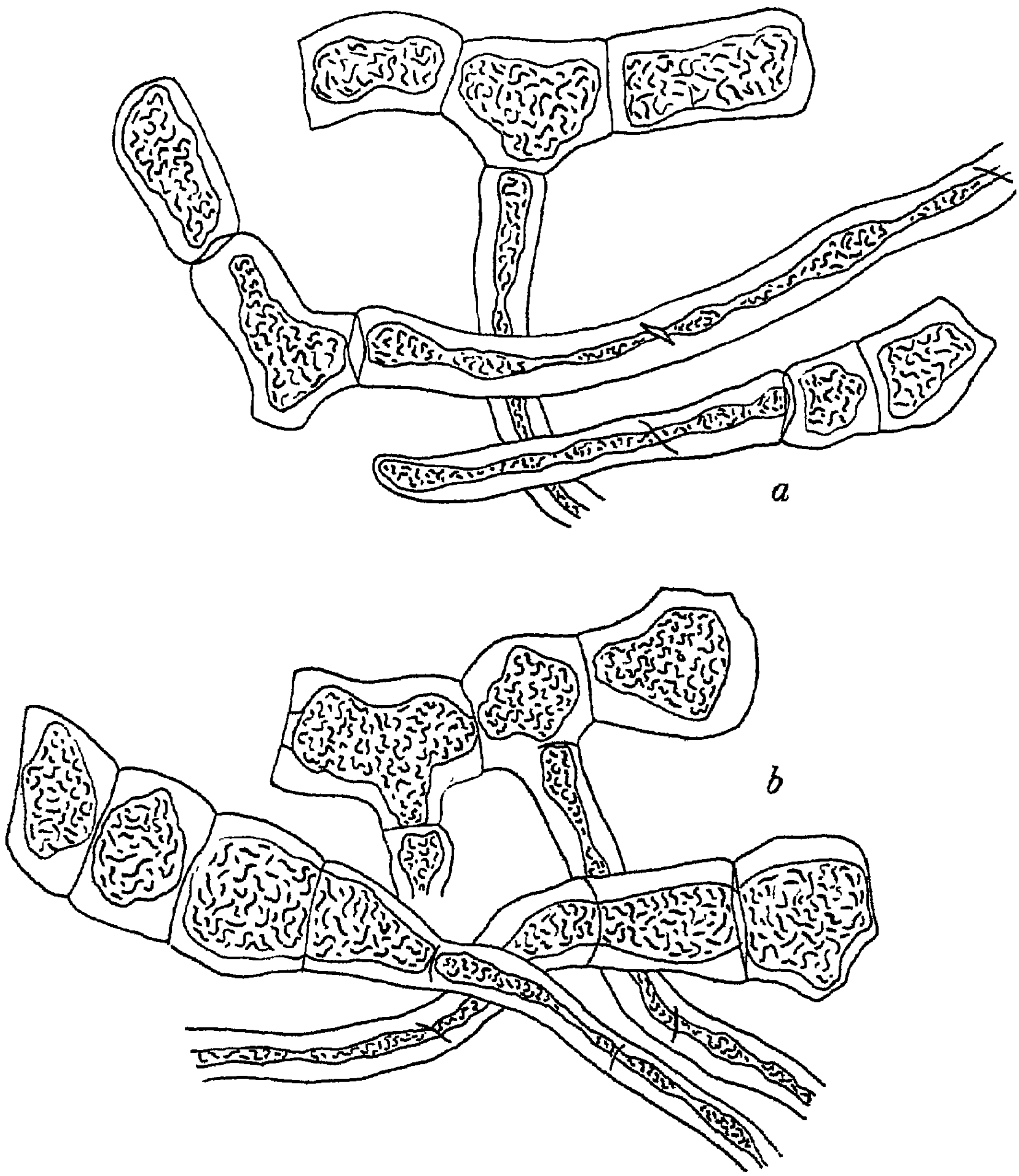
The growth of the hypha is apparently due to the activity of its apical cell, which is rich in protoplasm and has a thin-walled, rounded tip (Fig. 4*a*). Branching of the hyphae after they are once fully formed has only been observed in the holdfast tissue (Fig. 6*c*).

In a series of transverse sections from the tip of a branch downwards, the first hyphae appear within 1 cm. of the tip, in the medulla of the wings where it adjoins the compact midrib medulla. Almost immediately below this, hyphae become frequent on the borders of the medulla in both the wings and midrib, and very soon they appear within the compact medullary tissue of the midrib. At lower levels, the number of hyphae everywhere increases rapidly, but especially in the midrib, where at lower levels the strings of medullary cells are isolated by the masses of hyphae.

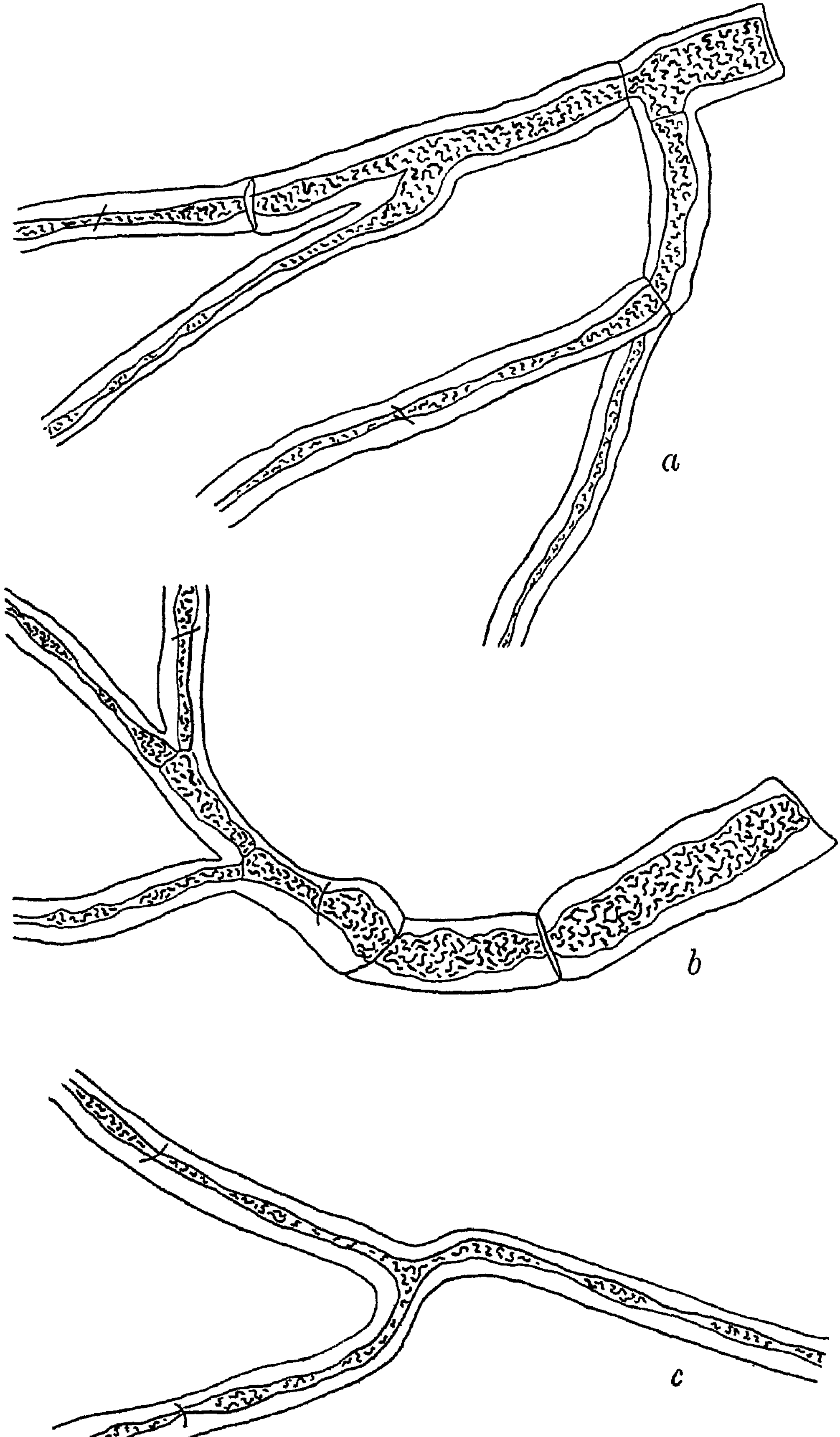
The origin of the hyphae is most easily seen in macerated and teased preparations. It is difficult to see in sections because the cells do not keep to one plane, but it can occasionally be seen in longitudinal section. Hyphae were found to originate from the following types of cell:



Text-fig. 4. *F. spiralis*, origin of hyphae at 1 cm. below apex of a full-grown plant, ×470. *a*, from cells of transition zone; *b*, from medullary cells.



Text-fig. 5. *F. spiralis*, origin of hyphae from transition zone in midrib, ×470. *a*, 1 cm. below apex of a full-grown plant; *b*, in the stipe.



Text-fig. 6. *F. spiralis*, origin of hyphae from medullary cells, ×400. *a*, from midrib, 10 cm. below apex of a 20 cm. plant; *b*, from stipe; *c*, branching hypha in holdfast.

(1) *Medullary cells in the wings.*

At about 1 cm. behind the tip of a branch, hyphae arise from strings of long narrow cells in the medulla of the wings (Fig. 4*b*). They apparently grow gradually into the midrib and continue downwards. Hyphae continue to arise from these cells in the older regions until the wings disappear.

(2) *Cells of the transition region between medulla and cortex.*

Almost simultaneously with those from the medullary cells, hyphae originate in the transition region of both wings and midrib. The cells of the transition region are shorter and wider than medullary cells, but give rise to hyphae in exactly the same way, by a filament of cells extending apically and giving rise to cells gradually more slender, until it becomes a typical thick-walled hypha (Fig. 4*a*). In the transition region, hyphae may also arise by lateral outgrowths from these strings of cells (Fig. 5*b*).

Hyphae continue to arise in the transition region, down to the base of the plant, but a change in the character of the cells in this zone becomes apparent in the region where the secondary cortex arises. The cells become shorter and almost cubical, apparently from transverse divisions like those occurring in the inner cortex at this level (Fig. 3*b*). They continue to give rise to hyphae as described above (Fig. 5*a* and *b*).

(3) *Medullary cells of the stipe.*

The origin of hyphae from the medullary cells of the stipe is first found in its typical form between 5 and 10 cm. behind the apex. Essentially it is the same process as that described above, i.e. the formation of a branch filament which grows down in the mucilaginous matrix, producing successively cells of smaller width until they have the typical appearance of a hypha. The frequent occurrence of a tuft of hyphae, due to repeated lateral branching of a medullary filament near its end, gives a characteristic appearance. Hyphae continue to arise in this way throughout the midrib and stipe, and in the holdfast, the cells of which are like those of the medulla (Fig. 6*a* and *b*).

The fertile branch

The hyphae described above are those from an ordinary vegetative branch, but hyphae resembling them in all respects have been found in the tip of a fertile branch.

The young plant

In a plant of less than 6 cm., the young branches are much more slender than are those of the same age in an older plant. The number of primary cells, especially in the medulla, is much less, as seen in transverse section. The development can be followed by comparing a transverse section about 5 mm. from the base of a 1 cm. plant with sections 5 mm. from the base of progressively older plants.

In the course of development, the number of parenchyma cells in a transverse section of the midrib is increased until they are about as numerous as in a branch of a full-sized plant. This takes place by cell division in the inner cortex adding cells to the existing medulla. This process can be regarded as a delayed continuation of the primary tissue formation.

The other peculiar feature of the young plant is that the first descending hyphae are formed much nearer the apex than they are in the mature plant.

DISCUSSION

The above investigation has shown that in *Fucus* all types of inner cells, and not, as had been supposed, just one particular layer, contribute to the secondary thickening by forming hyphae.

Secondary thickening by the addition of hyphae is of wide occurrence in the algae. It occurs in filamentous types like *Callithamnion* and *Bryopsis*, where the down-growing hyphae, whose appearance suggests a rhizoidal nature, corticate and strengthen the main axis, and also in algae of "cable construction", e.g. *Polyides* and various Ectocarpales, where the hyphae, in addition to growing downwards, grow inwards from the cells of the outer layer into the central "medulla".

Hyphae are found in parenchymatous algae of the most diverse construction and ontogeny—e.g. near the base of the *Ulva* thallus, between the two layers; in the older stipes of *Delesseria*, and as a lining to the tubular thallus of *Chorda*—algae whose only resemblance to *Fucus* is in the comparatively massive thallus, with cells separated by soft mucilage.

It seems probable that rhizoidal hyphae tend to originate wherever the construction of cells separated by soft mucilage makes possible their development, and entirely independently of the ontogeny of the tissue. The phylogenetic stages through which the tissue passed may

also be immaterial, as the same stimuli which cause and control hyphae formation in one thallus are likely to act where there is similar tissue in another.

Summary

1. The mature structure of *Fucus* is reviewed, and the secondary thickening by the formation of downward growing hyphae is discussed in detail.

2. The hyphae are filaments of slender cells which are shown to originate as lateral branches of cells in primary tissues. They grow apically, and may either be narrow along their whole length, or may form a series of gradually diminishing width down to that of the typical hypha.

3. All the internal cells may give rise to hyphae—both the cells of the inner cortex, and those of the medulla of wings, midrib and stipe.

I wish to thank Prof. T. M. Harris for suggesting this problem, and for his valuable help and criticism in the preparation of this paper.

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REVIEWS

Oekologie der Ackerunkräuter der Nordostschweiz. By M. BUCHLI. 9×6 in. 354 pp. (Beiträge zur geobotanischen Landesaufnahme der Schweiz, Heft 19.) Hans Huber, Bern, 1936. Price fr. 9.80.

Until comparatively recent years information relating to weeds, though extensive, has been chiefly derived from casual and scattered observations, uncorrelated with one another. During the last 25 years more intensive study has been undertaken, and attempts made to work out the connexion between weeds, soils and crops from various aspects The present treatise gives an elaborate analysis of the weed relations under the improved three-course rotation now practised in various parts of Switzerland, and provides a mine of information for future workers in the same field Many of the conclusions reached are not new, but owe their value to the support given by the results of detailed, quantitative work to the more vague assumptions of earlier observers and of practical men.

The first part of the book gives a detailed description of the districts investigated, with notes on climate and soil, methods of cultivation and the crop rotations. The general occurrence and distribution of the weeds is then described, followed by a discussion of the interrelation between the weed flora and such factors as rotation, soil dispersion and water capacity, and the reaction of the soil. Finally the question of number and viability of weed seeds present in the soil is dealt with, with special reference to the control of weeds by the destruction of seedlings.

The working scheme adopted is the usual one of listing the weeds present on a number of fields, with estimations as to their relative frequency. In addition, analyses of various kinds, both mechanical and chemical, together with information as to the crop rotations, provide the basis for numerous correlations. Altogether 205 weed species were observed on 274 fields, *Myosotis arvensis* being the most widely distributed. Interesting comparisons are made with Volkart's observations in mountainous districts, the frequency distribution of various common weeds being radically different in the two habitats, a fact which indicates the necessity of avoiding anything approaching dogmatism in considering the behaviour of any single species

The correlations are of many kinds, including the distribution of weeds according to the clay content of the soil, the chalk content, its acidity or alkalinity, and the size of soil particles, in addition to the association of weeds with various crops, both as individuals and as members of rotation. Unfortunately no index is provided, so that it is very laborious to trace any particular species through the different correlations, and the provision of such an index would much enhance the value of the book. Full use has been made of other work, and the various tables comparing the results show clearly how weed distribution is determined by a combination of factors of which perhaps one may be more important than the rest.

A fundamental feature in weed distribution is seed production and the accumulation of viable seeds in the soil. Enormous numbers of seeds can be produced, 143,404 having been recorded from a single plant of *Artemisia vulgaris*. The soil content of seeds can be determined directly by separating out the seeds, or indirectly by allowing the seeds to germinate and counting the seedlings. The latter method is preferable, provided sufficient time is allowed, as many seeds need to be dormant for some time before they are capable of

germination. Periodicity of germination has an important bearing on eradication, as cultivation must be done at the time that any particular weed species germinates most freely. The investigation led to the conclusion that a suitable rotation is the best background for all measures of weed control, with stubble working as a major preventive measure for most annual weeds, and draining as a useful means of reducing perennials

A good bibliography, from which, however, some of the more recent papers are omitted, concludes a volume which should prove of the greatest value to all who are interested in the question of weeds and their relation to crops and soils.

WINIFRED E. BRENCHLEY

Ergebnisse der Enzymforschung. V. Band. Edited by F. F. NORD and R. WEIDENHAGEN. 9×6 in. 378 pp., 16 text-figures. Akademische Verlagsgesellschaft M.B.H. Leipzig, 1936. Price: Brosh. M.28.50; geb. M.30.

The results of researches on enzymes are scattered in journals of biochemistry, physiology and medicine, so that it is difficult to keep track of the literature This led Prof. Nord and Prof. Weidenhagen to publish, each year, a collection of reviews of such work, and the first volume of *Ergebnisse der Enzymforschung* was issued in 1932. The editors made it clear that a series of impersonal summaries of the literature was not their aim. On the contrary, each contribution should be made by a specialist and should include his own views

Vol. v contains twelve contributions, of general or of special interest, from laboratories in Germany, Sweden, America, Holland and Britain. The practical and theoretical significance of the enzymatic production of optically active substances by plant and animal organisms is stressed by W. Kuhn in a paper on "Optische Specificita von Enzymen", and by A. McKenzie writing on "Asymmetric Synthesis". Kuhn emphasizes the importance of thermodynamic and kinetic analysis of the conditions for the formation of optically active substances. McKenzie gives a comprehensive account of asymmetric syntheses which have been brought about either by means of compounds of known constitution or enzymatically. An excellent account of the proteases by W. Grassman and F. Schneider brings up to date the original review of Grassman in vol. I. After brief reference to the scheme of classification the authors describe the preparation of proteases. The general structure of the enzymes is discussed and evidence is given for the sulphydryl nature of the active group in papain. The specificity of the peptidases in particular is considered in some detail in its bearing on the nature of the combining groups in the substrate. H. Haehn discusses the complex interplay of enzymes, activators and inhibitors during autolysis, when not only nutritional substances but also the building materials of the cell are broken down. Data are given for the hydrolysis of the chief classes of compounds during autolysis of animal organs and of certain plants Recent work on the phosphatases is reviewed by S. J. Folley and H. D Kay in a comprehensive paper which includes discussion of the biological significance of phosphatases. There is little exact knowledge of pectic enzymes, largely because the composition and structure of the pectic materials is unknown and their nomenclature unsettled, but Z. I. Kertesz makes it clear that pectinase is a complex of specific enzymes acting on certain linkages. The identity of protopectinase and pectinase is not established. Two comparatively short papers deal with oxidizing enzymes. D. Muller describes the glucose-oxidase of *Aspergillus* and *Penicillium*, an enzyme which is specific for the direct oxidation of *d*-glucose, *d*-mannose, and *d*-galactose, and has aroused a good deal of interest among plant physiologists. Muller considers glyconic acid to be an intermediate

product in the breakdown of glucose by these fungi. The mechanism of the action of polyphenol oxidases and their importance in plants is considered in a paper by H. Sutton. I. Smedley-MacLean treats of the various stages of the biochemical synthesis of fats from carbohydrates, a synthesis which has been proved for the animal body and is often assumed for plants though with little direct evidence. It is perhaps surprising to find included in this volume R. Emerson's Review of "Recent Investigations in the Field of Chlorophyll Photosynthesis", especially as rather more than half of it is devoted to work on rate measurements. The aim of this paper is the correlation of these results with chemical studies on chlorophyll. Finally, B. J. Krijgsman shows how the nephelometric technique may be used to measure the rate of disappearance of a substrate, e g. protein, glycogen, whose large, inactive molecules do not lend themselves to exact chemical methods. M. CATTLE

Les Bases scientifiques de l'Amélioration des Plantes. (*Encyclopédie biologique,* XIII.) By F. BŒUF. 543 pp. 46 text-figures. Paris: Lechevalier. 140 fr.

M. Bœuf's work forms part of a biological encyclopaedia. It covers more ground than any similar account, including the most general considerations of the "biosphère" and ranging from the smallest details of cytology to the largest problems of statistics and evolution. Such a work is not likely to satisfy the specialist in any one of these studies. We might, however, expect either accuracy of detail or originality in the integration of this comprehensive material. The author, it must be said, is disappointing in both these respects. His details are a patchwork of new and old, mutually disagreeing. The old story of *Primula kewensis* is combined with the new story of *Nicotiana digluta.* On p 108 intraspecific hybrids are of no cytological interest, but on p. 239 the cytological properties of 550 such hybrids are referred to with due consideration. Behind the mass of detail the author sketches a philosophical background which is in some ways original. He tells us that "Les organismes vivants, comme la matière inerte, tendent vers un état d'équilibre de plus en plus stable". Such statements go well with the cytological fairy tales of 1910, but they will neither of them provide a scientific basis for the amelioration of plants.

The bibliography is scattered and incomplete; about a quarter of the foreign names are misspelt in the text; there is no index.

C. D. DARLINGTON

THE

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ON THE NATURE OF CORRELATIVE INHIBITION

BY R. SNOW
Fellow of Magdalen College, Oxford

(With 5 figures in the text)

(1) AUXIN AND CORRELATIVE INHIBITION

A BIG advance was made towards the understanding of the correlative inhibition of buds and shoots when Laibach (1933), and independently Thimann & Skoog (1933, 1934) and Skoog & Thimann (1934), showed that the lateral buds of decapitated seedlings of various Leguminosae can be inhibited by a source of auxin, or of hetero-auxin, placed on the upper cut surface of the main stem. Especially, Thimann & Skoog (1934) have made a very valuable quantitative study of this process and of the effect of auxin on the growth of the main stem in *Vicia Faba*. These results place it beyond doubt that normally the rapidly growing leaves in and near the terminal bud, which are the parts that inhibit the lateral buds below (Snow, 1929*b*), do so by means of the auxin which they have been shown to secrete (Thimann & Skoog, 1934). The results thus confirm a previous conclusion by the writer (1925) that one stage in the transmission of inhibition is the movement of a special soluble substance down the main stem.

But there still remains the question how it is that the auxin descending the main stem inhibits the lateral buds, though it tends to promote the growth, both in length and thickness, of the main stem itself (see for instance Thimann & Skoog, 1934; Snow, 1935). This is essentially not a new question, since the writer (1932) discussed the same question previously in terms of the growth-regulating influences which were known to originate from the growing leaves, before it was known that these influences were due to the auxin

which these leaves secrete. For it was there discussed how it is that the growing leaves tend to promote the growth of their own shoot below them, but to inhibit lateral buds and also other shoots. An answer was proposed, based on various experiments, but only in rather general terms. In the present paper, therefore, it will be simpler to discuss the question in the more definite terms which have been made possible through the discovery of inhibition by means of auxin.

As to the question, then, how it is that auxin descending a stem brings about the inhibition of lateral buds, there are three main theories, the "direct" theory of Thimann & Skoog (1933, 1934), the "indirect" theory of Laibach (1933), which is essentially the same as a theory put forward by Loeb (1924) and which has also something in common with the conclusion reached previously by the writer (1932), and a recent theory by Went (1936) which may be called a "diversion" theory. These theories will here be discussed in turn in the light of various experiments, some old and some new.

(2) THE "DIRECT" THEORY AND EVIDENCE AGAINST IT

Thimann & Skoog (1934, p. 336) consider that an excess of auxin is secreted by the terminal bud, and that some of this auxin passes into the lateral buds and inhibits them by preventing them from forming auxin on their own account. This suggestion involves the difficult assumption that auxin reaching buds from elsewhere affects their growth differently from auxin which they make themselves. A rather different suggestion would be that the auxin inhibits the lateral buds because its concentration is too high.

But both these suggestions are open to the following objection. Since the auxin promotes the growth of the main stem in passing down it, it would be necessary to suppose that only *buds* are susceptible to inhibition by auxin reaching them from elsewhere (or by auxin in so high a concentration), and that they react to auxin differently from elongating stems. But an explanation cannot be reached along these lines, since in certain circumstances the growth not only of buds, but also of quite long shoots of pea and bean plants, including the elongation of their internodes, can be inhibited by other growing shoots (Mogk, 1913; Snow, 1931*b*). The growth of shoots can also be inhibited by a hetero-auxin paste applied below the elongating region (Le Fanu, 1936; Snow, 1936), although hetero-auxin applied from above promotes growth in length, unless the shoots already contain enough auxin (Thimann & Skoog, 1934; Le Fanu, 1936).

The writer's previous experiments (1931 *b*) with "two-shoot" plants seem indeed impossible to explain on the theory that inhibition is due to too high a concentration of auxin. In these experiments the main shoot of a young pea or broad bean seedling was removed, so that the axillary buds of the cotyledons grew out and formed two roughly equal shoots. Then one of these shoots was deprived of its rapidly growing leaves (of length 1·5 mm. and over) and it was found that this defoliated shoot was rapidly inhibited by the other shoot: for it soon stopped growing and finally died, whereas if the other shoot was removed, then the defoliated shoot grew on unchecked. Yet the removal of the rapidly growing leaves, which are the chief sources of auxin, must have *diminished* the supply of auxin—not increased it. This experiment will be further discussed in § 5.

It might indeed be suggested that the correlative inhibition of shoots is brought about differently from that of buds, and that the "direct" theory applies only to the latter. But the writer has never found any indication that there is any such difference, and it seems very unlikely that there is, since there is no sharp distinction between a bud and a shoot.

Again, it has recently been shown by Snow & Snow (1937) that hetero-auxin (which acts in practically all respects in the same way as the natural auxin), if applied in lanoline directly to part of the actual stem-apex of *Lupinus albus*, does not inhibit the formation of leaves and axillary buds from that part, but promotes it.[1] It is therefore not correct to say that auxin promotes the growth of shoots, but inhibits that of buds. For the direct effect of auxin on buds, when applied from above, is to promote their growth, while in certain circumstances auxin can cause shoots, as well as buds, to be inhibited. What these circumstances are will be made clear in § 4.

Evidence against the "direct" theory has also been provided by Le Fanu (1936), who has shown that in pea plants pieces of stems from inhibited shoots secrete no auxin, when tested by being placed on decapitated coleoptiles, although pieces from growing shoots secrete plenty. The writer has confirmed these results. Yet it would be expected that if the inhibited shoots had been inhibited by auxin

[1] Thimann & Skoog (1934, p. 337) state that in any case there is no evidence that the *initial* stages in the formation of buds are susceptible to inhibition brought about by auxin. But on the contrary they will find in an experiment by the writer (1931 *a*, p. 220) evidence that the initial stages in the formation of axillary buds are indeed susceptible to correlative inhibition, although normally, of course, these stages are not inhibited. An explanation of this paradox has been indicated previously by the writer (1931 *a*, p. 221).

passing into them, then the auxin would be able to pass out of them again. Since it does not do so, one would have to assume, on the "direct" theory, that it is all somehow inactivated or retained in the inhibited shoots, which does not seem very likely.

Another difficulty pointed out by Le Fanu (1936) is that the auxins in shoots are transported only comparatively feebly in the morphologically upward direction in *Pisum* and in *Vicia Faba* (Thimann & Skoog, 1934, p. 337), though they are indeed transported upwards to some extent in *Pisum*, as she and the writer (1936) have found. Yet inhibition can travel upwards for considerable distances in a decapitated shoot of a "two-shoot" plant of either of these species (produced by removing the main shoot and allowing the cotyledonary buds to develop), as the writer has shown (1929*a*, 1931*a*, *b*). Indeed, in decapitated pea shoots of this kind the writer has found recently that inhibition can travel upwards for distances of from 10 to 15 cm. at least, and there was no sign that it was weakened in so doing.

Thimann & Skoog (1934, p. 338) seek to explain such facts by suggesting that some auxin travels upwards in side-shoots with the transpiration stream in the vessels. In support they refer to an experiment by the writer (1929*a*) in which an inhibiting influence travelled up with the transpiration stream through a dead zone of a side-shoot. But in this experiment, as was pointed out (1929*a*, p. 264), dead vessels were in contact with living tissue, so that the conditions were peculiar. The following experiment shows that inhibition can travel in the morphologically upward direction even against the direction of the transpiration stream.

Seven pea seedlings, each with two nearly equal cotyledonary shoots, were dug up and deprived of their root systems. One of the two shoots of each seedling was decapitated just below the second leaf from the base, and the decapitated shoot was placed inverted with its upper cut end in a little water in a glass tube (Fig. 1). The second shoot was left intact in four of the plants, and in the other three, which served as controls, it also was decapitated.

After 6 days (in June, in the shade) in the three controls the axillary buds at the first leaf-nodes of the shoots in the tubes had grown 17, 11·5 and 9·5 mm., whereas in the four experimental plants these buds had not grown at all, so that they must have been inhibited completely by the intact growing shoots. In these four plants, therefore, inhibition travelled up from the cotyledonary nodes to the first leaf-nodes of the decapitated shoots over distances

of 25 or 30 mm., although the transpiration stream was moving in the opposite direction.

The conclusion that the inhibiting influence can travel where auxin does not travel can also be reached by relying on the fact that auxin when present, even in very low concentrations such as are secreted by growing buds, activates cambial growth (Snow, 1935; Gouwentak, 1936).[1] For the inhibiting influence can travel up into defoliated side-shoots in *Vicia Faba,* though such shoots make no

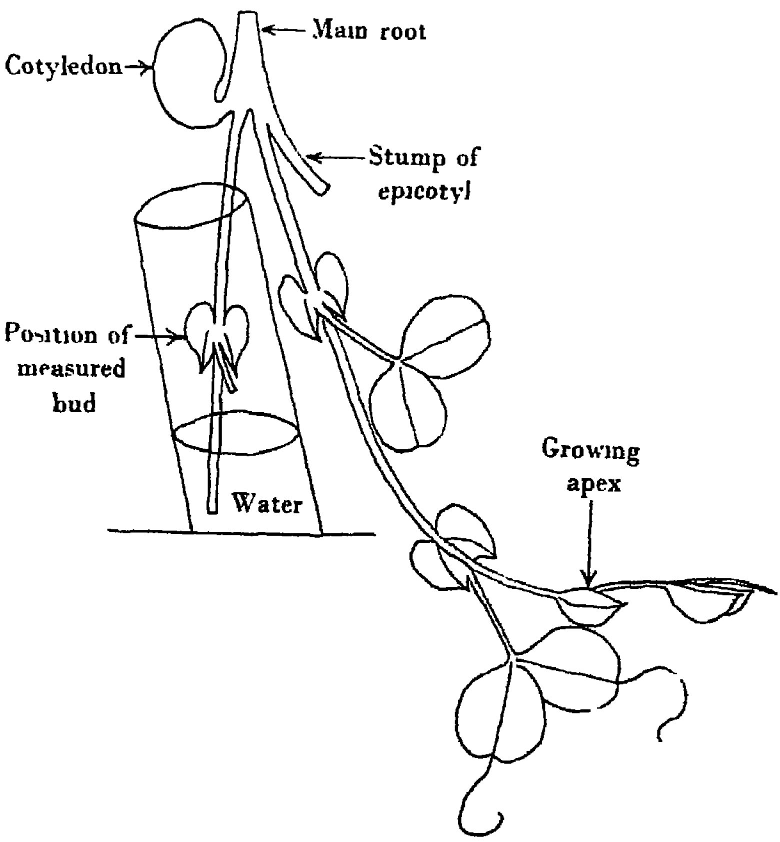

Fig. 1. The measured bud is inhibited.

cambial growth, but turn limp and die after 2 or 3 weeks (Snow, 1931*b*). Indeed, in shoots of *Vicia Faba,* cambial growth is very strictly limited to those stem parts which can be reached by influences travelling in a morphologically downward direction from leaves and growing buds above, as the writer has found previously (1932, pp. 100 and 90).

[1] Went (1936) has asserted that auxin stimulates cambial growth only in high concentrations, but his assertion is contrary to the evidence, since the writer (1935) and Gouwentak (1936, p. 17) have shown that cambial growth can be stimulated by very small amounts of auxin, roughly equal to those which are secreted by growing buds.

It may indeed be objected that possibly auxin may be able to travel upwards, but may yet for some unknown reason fail to activate cambial growth when so doing. But the following experiment is not open to this objection: it also provides evidence against the "diversion" theory, to be discussed below. It can be understood most easily from Figs. 2 and 3, which show one experimental plant and one control as they appeared at the end of the experiment. For these two drawings I am indebted to my wife. The arrangement was the following.

Young seedlings of *Vicia Faba* (Johnson's long pod) were dug up and split through the cotyledonary node, with a median longitudinal split which was continued downwards right through the main root. They were then replanted, and later the split was continued upwards through the epicotyl nearly to the first node, and the main shoot was decapitated higher up, above the third leaf which was also removed. When the two buds in the axils of the cotyledons had grown out and formed shoots, one of these shoots in each plant was decapitated above its first leaf, in order that it might be seen whether the bud in the axil of this leaf would be inhibited by the other cotyledonary shoot across the zigzag path of tissue connecting the shoots. In controls both cotyledonary shoots were decapitated.

In a first series, in November, in four experimental plants the buds in question grew in 17 days only 1, 1·5, 2 and 6·5 mm., while in five control shoots they grew 6·5, 7, 9, 12 and 14 mm. In a second series, in December, the buds in four experimental plants grew in 18 days 0·25, 0·5, 1·0 and 1·5 mm., while in four control shoots they grew 4, 5, 7 and 8·5 mm.

The cotyledons were not exhausted at the ends of the experiments. The temperatures were very low, and this accounts for the fact that even in the controls the buds grew out only very slowly.

It can be seen that in seven out of eight experimental plants, the measured bud was nearly completely inhibited. The inhibiting influence must have reached this bud from the other cotyledonary shoot after travelling up and down again through the halves of the split epicotyl, the length of each of these halves being from 7 to 10 mm. Yet there was no sign of cambial growth on the cut surfaces of these halves of epicotyl, although in the farther half, if there had been any auxin coming from the growing cotyledonary shoot, it would have been travelling morphologically downwards.

It might indeed be objected that perhaps the experiment did not last long enough for cambial growth to show itself. But in a previous

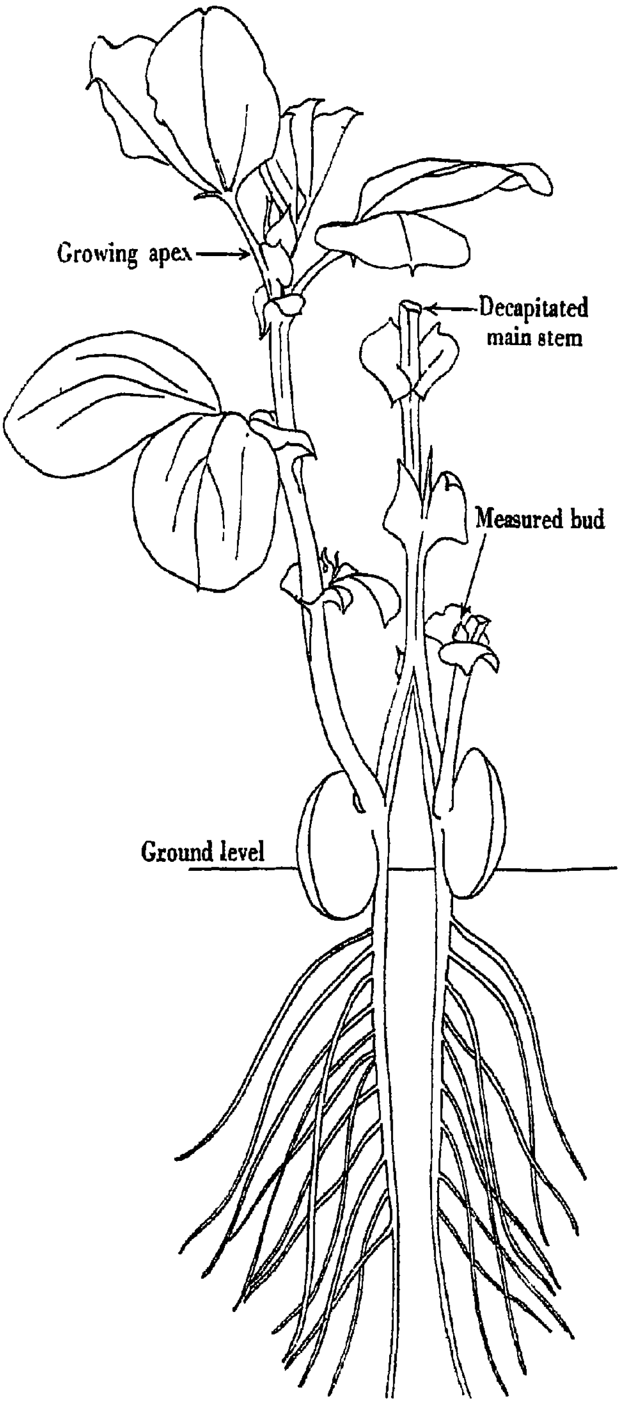

Fig. 2. The measured bud is inhibited.

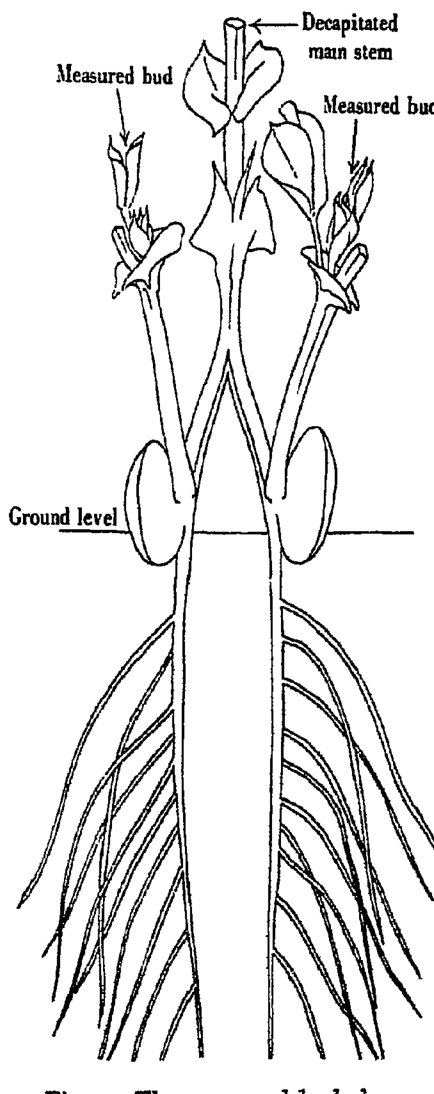

Fig. 3. The measured buds have grown out.

experiment the writer (1932, Exp. 5, p. 96) grew for 2 months or more in full summer several plants of *Vicia Faba* which in all relevant respects had been operated on in the same way,[1] and in these also

[1] Actually the only difference in the earlier experiment was that the decapitated cotyledonary shoot was cut off at the base, instead of above the first node.

there was practically no cambial growth in the epicotyl halves as microscopic examination showed, though they remained alive all the time (see (1932), p. 99). Yet the halves of split stems of *Vicia Faba* do make very conspicuous cambial growth in much shorter periods if they are so situated that they can be reached by the auxin travelling in the morphologically downward direction from leaves or growing buds above them, even if those leaves and buds belong to another (lateral) shoot, as the writer has shown in the same paper (1932, pp. 100 and 90).

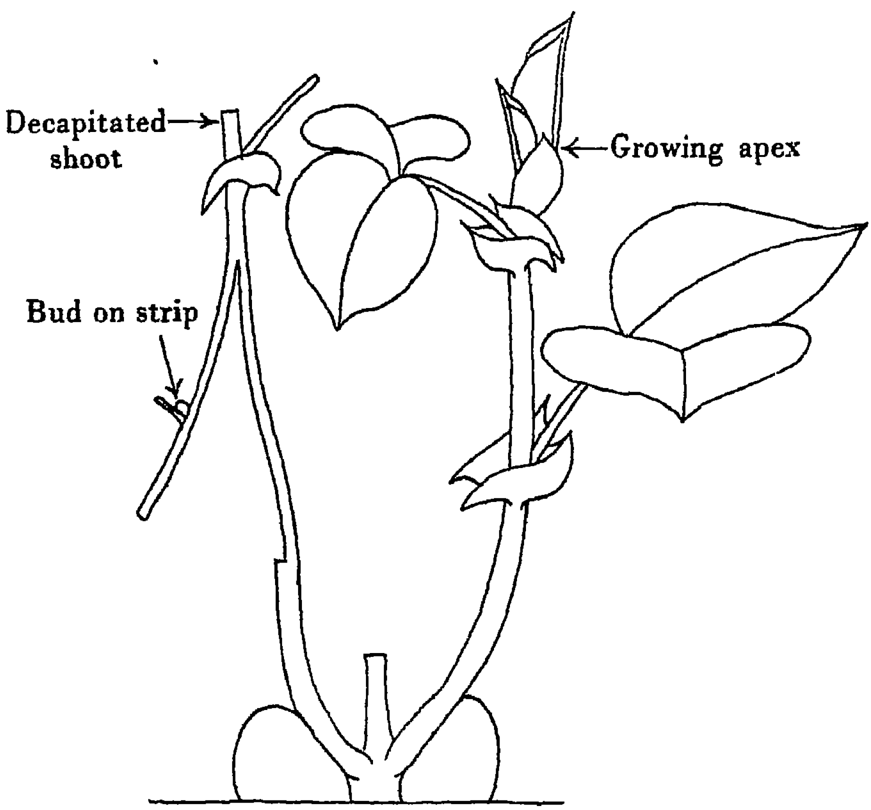

Fig. 4.

The conclusion that the inhibiting influence can travel where auxin cannot travel can be reached much more simply, but perhaps a little less decisively, by the experiment illustrated in Fig. 4, in which one shoot of a "two-shoot" plant of *Vicia Faba* is decapitated and then has a downward-pointing strip cut out from it. For any axillary buds that are present on such downward-pointing strips are completely inhibited by the other shoot of the same plant, as the writer has repeatedly found: but neither the downward-pointing strips nor any part of the decapitated shoots make any appreciable cambial growth. It follows that no auxin, or hardly any, can be reaching the downward-pointing strips, for similar strips of stem in

Vicia Faba make vigorous cambial growth if cut out from growing shoots, in the manner shown in Fig. 5, so that they have growing leaves above them (see Snow, 1932, p. 90). With this arrangement

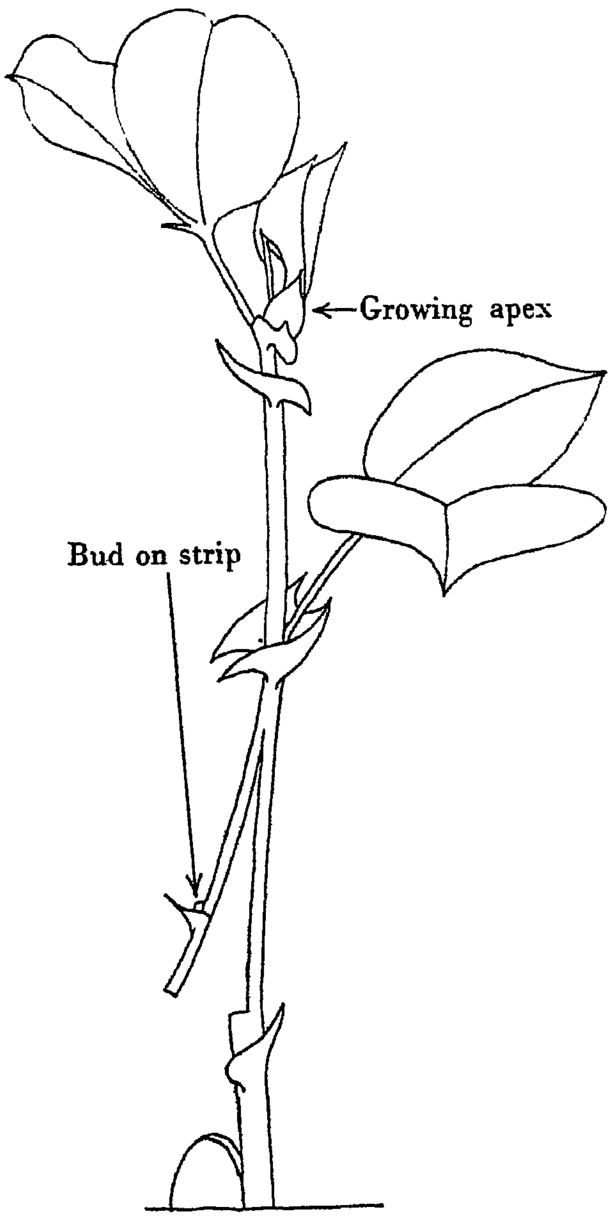

Fig. 5.

also the buds on the downward-pointing strips are completely inhibited.

The evidence brought forward in this section seems to the writer to show that the "direct" theory cannot explain the correlative inhibition of buds and shoots.

(3) THE "DIVERSION" THEORY OF INHIBITION

Went (1936) has tried to explain the correlative inhibition of lateral buds by supposing that there are some special substances which come up from roots and cotyledons and which are necessary for the growth of buds. His full evidence for the existence of these substances is promised for a later paper. He further supposes that the auxin coming down from the terminal bud and leaves polarizes the main stem in such a way that the substances necessary for bud growth travel up it towards the source from which the auxin is coming, and do not pass out into the lateral buds which consequently do not grow.

This theory seems at first rather plausible, but there are several facts which it cannot explain. Firstly, it cannot explain the result of the experiment illustrated in Figs. 2 and 3 (above). For in that experiment the inhibited bud on the decapitated cotyledonary shoot cannot possibly have been deprived of any substances coming up from the half root-system and cotyledon below that shoot through any polarizing action of the auxin descending from the other cotyledonary shoot into the other half root-system, as a glance at Fig. 2 will show.

Again, one can see that Went's theory is inadequate if one remembers that when a pea or bean seedling possessing two cotyledonary shoots has one of the shoots decapitated, then the lateral buds on that shoot are still completely inhibited by the other shoot (Snow, 1929*a*, 1931*b*). Now on Went's theory one might perhaps seek to explain this fact by supposing that substances necessary for bud growth are so directed that they all travel up from the root system into the growing shoot, and do not enter the decapitated shoot at all. But this explanation cannot be correct, since, in the light at least, the lateral buds simply do not need any substance coming from roots or cotyledons in order that they may start to grow out, except only water of which the movement does not depend on any polarizing effect such as Went postulates. For if one cuts out from a pea shoot a short piece of stem including a single node with its leaf and axillary bud, and if one places the cutting with its base in distilled water and exposes it to light, then the axillary bud grows out after a few days, as Le Fanu (1936, p. 207) and the writer have found. Consequently Went's theory cannot explain the complete inhibition of the buds on a decapitated side-shoot.

Admittedly the buds on cuttings of pea shoots in distilled water do not grow to lengths of more than 3 or 4 cm., as the writer has found: for then the cuttings turn yellow and die, so that for the further growth of the buds some other nutritive substances appear to be needed, as would be expected. But still the buds on these cuttings do start to grow out, whereas on a decapitated side-shoot they are completely inhibited.

It would also be difficult to explain on Went's theory a previous experiment by the writer (1932, p. 93) in which downward-pointing strips of stem of *Vicia Faba* seedlings were cut out in a manner similar to that shown in Fig. 5, but from very young internodes, only 2·75 mm. long or less. For these strips elongated nearly as rapidly as the other halves of the young internodes, although, on Went's theory, they should have failed to receive some necessary growth substances coming up from roots and cotyledons. It may indeed be objected that these were strips of internode, and not buds. But the elongation of internodes is susceptible to correlative inhibition, just like the growth of buds (Mogk, 1913; Snow, 1931*b*): consequently if Went's theory were correct, these downward-pointing strips should have been inhibited, which actually they were not.

The facts mentioned in this section seem to the writer to show that Went's "diversion" theory cannot be both correct and adequate. If indeed there were various ways in which correlative inhibition of buds and shoots could be brought about in various circumstances, then possibly the diversion theory might account for one of them. But there is no indication that there is more than one way, and consequently it seems that, unless such evidence can be found, the theory must be rejected.

Yet it is very probable that *after* a bud or shoot has been inhibited, various solutes are diverted from entering it in some such manner as Went proposes. For this is made probable by the valuable experiments of Neeff (1914), which show that in a decapitated shoot the conducting cells near to the base of a growing side-shoot become reorientated towards the apex of that shoot.

(4) The indirect theory of inhibition

Laibach (1933) considers that auxin travelling down a main stem promotes its growth, and that the growth of the main stem then inhibits the axillary buds in some way not yet known. A similar theory was proposed by Loeb (1924, p. 101), who considered that in

Bryophyllum the apical leaves promote the growth of the main stem below them, and that the growth of the stem then inhibits the axillary buds.

On the basis of a theory of this kind, it is easy to suggest an answer to the question how it is that auxin tends to promote the growth of a stem down which it travels, but to inhibit lateral buds and other shoots. The answer is the following. In a stem down which auxin is travelling, the auxin with its tendency to promote growth overrides the secondary inhibiting influence and prevents it from producing its effects. Evidence that auxin in sufficient concentration does indeed prevent the inhibiting influence from showing itself will be given in the next section. But auxin, as is known, travels mainly (though not entirely) in the morphologically downward direction in stems, and consequently not much of it will travel upwards into a lateral bud or shoot. But the secondary inhibiting influence *can* travel in the morphologically upward direction, as one must suppose, and consequently it produces its effect in a lateral bud or shoot where there is not enough auxin present to override it. Thus on this theory the base of a lateral bud or shoot acts in a sense as a filter which allows the inhibiting influence (whatever it may be) to pass up into the lateral bud or shoot but prevents more than a little growth-promoting auxin from doing so. The correlative inhibition of a lateral bud or shoot, therefore, depends on its being separated from the main source of auxin by a zone of tissue in reverse orientation.

This is essentially the same conclusion as was reached previously by the writer (1932, p. 94 and pp. 102, 103), though at that time it was not known that the influences promoting stem elongation and cambial growth, which descend from the growing leaves, are due to the auxin which these leaves secrete (Thimann & Skoog, 1934; Snow, 1935). It was also left as an open question whether the secondary inhibiting influence originates from the growing leaves, or from the growth processes which these leaves promote in the stem below them (Snow, 1932, p. 103): but the second of these alternatives must now be preferred, in view of the recent discovery of inhibition by means of auxin.

If the "indirect" theory is developed in this way, it provides a simple explanation of the main problems of correlative inhibition, and, so far as the writer is aware, there are no known facts which are inconsistent with it, provided that it is modified in the following way.

Skoog & Thimann (1934) object that when they brought about the inhibition of the axillary buds in decapitated pea seedlings by placing a source of auxin on the top of the stem, they found that the stem did not grow in thickness. But they do not state that they examined the stems anatomically, so that there may have been a few cambial divisions stimulated by the auxin. A more serious objection is that there is strong correlative inhibition in the climbing shoots of *Tamus communis*, a monocotyledon, as the writer has found: for if the tip of the shoot is cut off, several of the upper axillary buds soon grow out strongly, though in intact shoots the axillary buds do not grow out. Yet in these shoots there is, of course, no cambial growth, and scarcely any primary growth in thickness (if any) except fairly near the tip. It therefore seems safer to suppose only that the secondary inhibiting influence originates from a positive primary process of some kind which is stimulated by the auxin in the stem down which it travels, without postulating that this primary process need always be growth. Perhaps it is some preliminary stage which according to circumstances may or may not be followed by actual growth.

Some other results which may at first seem a difficulty for the "indirect" theory have been reported by Dostál (1936), who put hetero-auxin paste on one of the cotyledons of a soaked seed of *Pisum* or of some other member of the Viciae, and found that the growth of the petiole of the cotyledon was strongly inhibited within the next 24 hr., although the hetero-auxin must have descended through it. But he himself (1936, pp. 216, 217) gives one reason for thinking that this inhibition was brought about in a different way from the correlative inhibition of axillary buds, and another reason for so thinking is that the petioles of underground cotyledons are positively geotropiclike roots, in some species at least, as Copeland (1903) has shown for *Aesculus Californica*; for this makes it probable that the growth of the petioles of these cotyledons is retarded by the auxins in the same manner as is the elongation of roots.[1]

Some new facts which can readily be explained on the "indirect" theory were reported recently by Le Fanu (1936) and by the writer (1936). Le Fanu applied hetero-auxin in lanoline to the stems of pea seedlings below the elongating region, and found that it retarded

[1] In *Pisum* also, as the writer finds, the short petioles of the cotyledons are slightly positively geotropic. They also show a curious effect which may be related. If the soaked seed (without testa) is laid on one side, the petiole of the upper cotyledon elongates, but the petiole of the lower one does not.

the elongation of the parts above, although the same paste applied from above accelerated elongation. The writer (1936) confirmed this result, and added the point that the strong and increasing retardation of the parts above the hetero-auxin paste is preceded by an acceleration during the first 12 hr. or thereabouts. These facts can be explained as follows. Above the auxin paste the preliminary acceleration is due to a little of the hetero-auxin travelling (with difficulty) upwards, and the retardation is an indirect effect similar to correlative inhibition. But when the paste is applied above the elongating zone, the hetero-auxin travelling down through this zone in higher concentration prevents the indirect inhibiting influence from producing its effect. These facts therefore tend to support the theory.

Le Fanu (1936, p. 207) found also that the elongation of young internodes of pea shoots cut off at the base was retarded by weak solutions of hetero-auxin drawn up with the transpiration stream. The writer (1936, p. 300) made observations which at first seemed to him to indicate that this retardation was probably an effect of a different kind, and was not to be explained on the basis of the "indirect" theory. But on further consideration it now seems that the facts reported might well be explained on the "indirect" theory after all. For the very young internodes (less than 6 mm. long) were the only ones which were inhibited by hetero-auxin in the transpiration stream, and these may have been retarded by a secondary influence originating from a direct positive effect of the hetero-auxin on the older parts of the stem below. The older growing internodes were indeed accelerated. But the question needs to be investigated further.

It has been pointed out already that the inhibiting influence can travel upwards in a side-shoot: that it can also inhibit the elongation of a strip cut out from a side-shoot, in which it is travelling in the morphologically downward direction, was shown previously by the writer (1932, p. 87), in an experiment which he has since repeated with better controls and confirmed. Correlative inhibition, therefore, does not depend on the morphological direction in which the inhibiting influence is travelling through a tissue, provided that this influence has once become separated from the auxin by travelling through a stretch of stem in the morphologically upward direction.

The conclusion reached in this section is that the "indirect" theory, if widened in the way suggested above, is right so far as it goes: but it may need to be still further widened, and the details need to be worked out. Le Fanu (1936) also concluded that the "indirect" theory was probably right.

(5) The protective influence of the young leaves

From previous experiments with "two-shoot" plants, which were mentioned in § 2, the writer concluded that the rapidly growing leaves tend not only to inhibit lateral buds and other shoots, but also to protect their own shoot below them from inhibition: for if these leaves are removed from one shoot of a "two-shoot" plant, then that shoot is rapidly inhibited by the other shoot. From subsequent experiments the writer (1932, pp. 94 and 102) concluded that this protective influence of the young leaves was the same as the various growth-promoting influences, including the cambial stimulus, which these leaves exerted on the stem below them. But it is now known that most, or perhaps all, of these growth-promoting influences are due to the auxin which the young leaves secrete (Thimann & Skoog, 1934; Snow, 1935), and consequently it is to be expected that it is also by means of auxin that the young leaves protect their own shoot from inhibition.

This expectation was tested in the following experiment. In seventeen pea seedlings, each with two cotyledonary shoots, one of the shoots was decapitated near the top of an internode, so that its growing leaves were removed. Then in nine of the seedlings the decapitated shoot was given a cap of hetero-auxin paste, of concentration 1 in 1500, on the upper end, and in the other eight seedlings it was left without paste.

When the intact shoots had grown for about three plastochrons, it was found that the upper internodes of the eight decapitated shoots without auxin paste were limp and dying, whereas in seven of the nine decapitated shoots with auxin paste they were still turgid and had elongated a little.

Auxin, therefore, tends to protect a shoot down which it travels from being inhibited by other shoots, and it may safely be concluded that it is by means of the auxin which they secrete that the growing leaves tend to protect their own shoot from being inhibited. This provides support for the assumption, proposed in the previous section, that auxin travelling down the stem of a growing shoot prevents the secondary inhibiting influence which originates in that stem from producing its effects there.

(6) THE NATURE OF THE INHIBITING INFLUENCE

The inhibiting influence must continually be entering an inhibited lateral bud or shoot and travelling up it, as follows from the facts reported in §§ 2 and 3. This influence according to the "indirect" theory is not the auxin, which travels down the growing shoot but hardly travels at all up into a lateral shoot, and the theory must therefore remain very incomplete unless the nature of this secondary inhibiting influence can be discovered.

Some evidence is provided in an experiment by the writer (1929*a*), who showed that in *Vicia Faba* an inhibiting influence can travel upwards with the transpiration stream through a dead zone of a decapitated side-shoot: for this was probably the secondary inhibiting influence, and if so, then this influence must be a soluble substance or substances of some kind.[1] But further evidence is needed, since it is possible, though unlikely, that in this experiment it was a little auxin which somehow got into the basal part of the side-shoot and was then drawn up through the dead zone and brought about the inhibition of the buds above.

Accordingly the writer has tried in various ways to extract from inhibited shoots some substance or substances to which the inhibition might be due, but up to the present without success. It would be necessary to show that such a substance was present in inhibited shoots, but not in otherwise similar non-inhibited shoots. The inhibited shoots were provided by decapitating one shoot of a pea seedling possessing two cotyledonary shoots, and leaving it for about a week to be inhibited by the other cotyledonary shoot (see § 5). An inhibiting substance can indeed be found in the expressed saps of many tissues, when crushed in water, as is well known: but this substance is apparently present in the saps both of inhibited and of non-inhibited shoots.

(7) SUMMARY

1. Thimann & Skoog (1933, 1934) and Laibach (1933) have shown that it is by means of auxin that the apices of growing shoots (or more strictly the growing leaves near the apex) inhibit the lateral

[1] The inhibiting influence can also travel otherwise than with the transpiration stream, as was shown in § 2, and it is not likely that it travels with the transpiration stream except when it can leak into the vessels near a dead zone (see Snow, 1929*a*, p. 264).

buds below: for the same inhibition can be caused by an artificial source of auxin placed on top of a decapitated main stem.

2. As to the manner in which the auxin brings about the inhibition of the lateral buds, there are three main theories—a "direct" theory of Thimann & Skoog (1934), a "diversion" theory of Went (1936), and an "indirect" theory of Loeb (1924) and Laibach (1933). These theories are discussed in turn in the light of various experiments, some old and some new, and a decision is reached in favour of the "indirect" theory with the following modification.

3. According to the "indirect" theory, the auxin travelling down a stem promotes its growth, and the growth of the stem then somehow inhibits the lateral buds secondarily. But reasons are given for thinking that the primary process promoted by the auxin in the stem need not always be actual growth.

4. On the basis of the "indirect" theory an answer can be suggested to the question how it is that the growing leaves near the apex tend to inhibit the lateral buds and also other shoots, although they tend to promote the growth of their own shoot. This answer, which is essentially the same as was reached previously by the writer (1932, p. 102), is briefly that in a stem down which auxin is travelling from the growing leaves the primary positive effect of the auxin overrides the secondary inhibiting influence. But not much auxin travels in the morphologically upward direction into a lateral bud or shoot, whereas the inhibiting influence does travel up into a lateral bud or shoot and so produces its effect there.

5. Evidence is given that auxin tends to protect a shoot down which it travels from being inhibited by other shoots: it is therefore by means of auxin that the growing leaves tend to protect their own shoot below them from inhibition (Snow, 1931 *b*), and this supports the "indirect" theory.

6. The nature of the secondary inhibiting influence is discussed.

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SOME NEW FEATURES IN THE REPRODUCTIVE CYTOLOGY OF ANGIOSPERMS, ILLUSTRATED BY *ISOMERIS ARBOREA*

By FREDERICK H. BILLINGS
University of Redlands, California

(With 51 figures in the text)

Introduction

From a development standpoint, the chief contributor to our knowledge of the Capparidaceae is Mauritzon (1935), who studied the development of the female gametophyte and the embryogeny of the following species: *Capparis frondosa*, *C. rupestris*; *Cleome monophylla*, *C. serrata*, *C. spinosa*, *C. violacea*; *Dactylaena micrantha*; *Gynandropsis pentaphylla*. In *Capparis frondosa*, however, he observed only post-fertilization stages, and in *C. rupestris* only those of pre-fertilization.

In the relatively large size of the nucellus there is general correspondence with *Isomeris*; also in the hypodermal position of the archesporial cell and the formation of several parietal cells. The seeds of all of the Capparidaceae are of course of the campylotropous type. These are the chief points of resemblance, but in other matters concerned with developmental history there is wide divergence. In the species studied by Mauritzon (1935) a group of four megaspores is quite regularly formed, the functional one developing into a megagametophyte of the normal type. In it there is a true egg nucleus, a group of two polars and one of three antipodals. None of these occurs in *I. arborea*, nor does fertilization, a process taken for granted in the text of Mauritzon's paper, though not definitely described or figured. In the species described by this author, embryos arise from the zygote and begin development at the micropylar tip of the megagametophyte. In *Capparis frondosa*, in addition to the normal embryo there originate from the nucellus about twenty adventitious embryos. The embryo in *Isomeris arborea* does not arise from a zygote or an egg cell, nor does it develop at the micropylar tip of the megagametophyte. There are no nucellar embryos in *I. arborea*. No proliferation of synergids such as that occurring at times in *I. arborea* is reported for any of the species studied by Mauritzon (1935). In *I. arborea* there is no suspensor haustorium. One is reported for *Polanisia trachysperma*. Finally, the manner of develop-

ment of pro-embryos in *Isomeris* is different from that described in the species listed by Mauritzon (1935). Unfortunately, this author does not list the chromosome counts of the species he had under observation.

Guignard (1893), though an early investigator of one of the species of the Capparidaceae, *Polanisia graveolens*, devoted his attention chiefly to the development of the seed coats.

The Capparidaceae are represented in California, according to Jepson (1925), by six genera and some twelve species. Some of them, including the single species of *Isomeris* found within our borders, are desert forms. *I. arborea* is found on both the Colorado and Mohave deserts, which lie a short distance to the east and to the north, respectively, of Redlands, California. There is upon the bush at all seasons a profusion of bright yellow flowers that greatly exceed proportionately the number of inflated pods that have arisen from the fertile flowers. The cause of the relatively small number of fruits is found in the presence of an aborted ovary in many of the flowers. The stalked ovary in fertile ones is situated well up among the anthers in the late bud stage. Bees are the principal insects that visit the plant, though cross-pollination does not necessarily occur, since a single bush, bearing seeds with mature embryos, has been found at least 30 miles distant from the nearest representative of the species. All flowers produce anthers, but there is a tendency toward degeneration in these organs, for many do not dehisce at all, while certain others discharge only a small amount of pollen.

Young buds intended for the study of meiosis were fixed in Carnoy's fluid, mounted on cardboard squares, embedded in nitrocellulose and cut on a sliding microtome. Material for embryo-sac development was embedded in paraffin. Nitrocellulose, however, was found to be very advantageous in cutting sections of young seeds.

MICROSPOROGENESIS

At the microsporocyte stage the archesporium is approximately 8 cells thick in median cross-section. An anomaly occurs in diakinesis when univalents only appear (Fig. 1). Since disjunction is impossible without synapsis, the daughter chromosomes that appear in the early anaphase of I, have arisen through equational division (Fig. 2). The chromosomes are very small (about 0·28 μ in diameter), approximately spherical in form, and number 17. Counts available for other Capparidaceae are as follows:

	n	2*n*	Authority
Capparis spinosa	(19)	38	Kuhn (1929)
Capparis rothii	20	—	Hagerup (1931)
Cleome paradoxa	16	—	Tischler (1921–22)
Cleome spinosa	(19)	38	Taylor (1925)

In *I. arborea* there is a count in both I and II of 17 (Figs. 3, 4), a somatic count of 17 (Fig. 5) and a megagametophytic count also of 17 which is shown by mitosis in its development (Fig. 20), and in that of the endosperm (Fig. 6). There is no true meiosis nor syngamy in the species. Indications that a state of haploidy exists are shown by the following facts: (1) there is an absence of bivalents in diakinesis; (2) the odd number, 17, is probably not a diploid number; (3) the *n* range thus far determined for the Capparidaceae, being 16–20, it is quite likely that 17 represents a haploid number. Hence *Isomeris arborea* doubtless finds a place among the haplodiplonts. Bleier (1933), who thus designates sporophytes which exhibit no diploid condition, has published a list of fifteen haplodiplonts of the "*Datura stramonium*" type (Sharp, 1934). This species, according to Belling & Blakeslee (1927), shows both an *n* and a 2*n* count of 12, the *Datura* monoploid number. Other species in the list show different monoploid numbers. It is in this group that *Isomeris arborea* would belong, but it is exceptional in that it appears to be the first instance of monoploidy in a wild, well-established species that has many individuals which look alike.

The second division (II), like I, is accompanied by equational splitting and equal distribution of the chromosomes to the poles (Fig. 7). In *Datura*, according to Belling & Blakeslee (1927), there is a random distribution of the chromosomes without splitting in I, but with equational division in II. In *Oenothera franciscana* and *O. hookeri*, Bleier (1933) reports that part of the chromosomes in I pass to the poles undivided, while others split. In *O. franciscana* 22 % of the chromosomes show no equational division in I, but pass at random to the poles.

Division into microspores in *Isomeris arborea* is simultaneous and is accomplished by true furrowing (Fig. 8).

The nucellus

There are four elongated parietal placentae in the ovary of *I. arborea* (Fig. 9). The cylindrical mound of tissue that represents the ovule primordium consists at first chiefly of funiculus, but at its tip a secondary papilla, which is young nucellus, arises subterminally (Fig. 10). The nucellus is scarcely more than a hemispherical mass, before a protuberance arises near its base, which is the fundament of the inner integument. The nucellus has two different regions of elongation, one at the tip, the other near the base. Elongation at the tip is by periclinal divisions of the hypodermal cells, and its degree

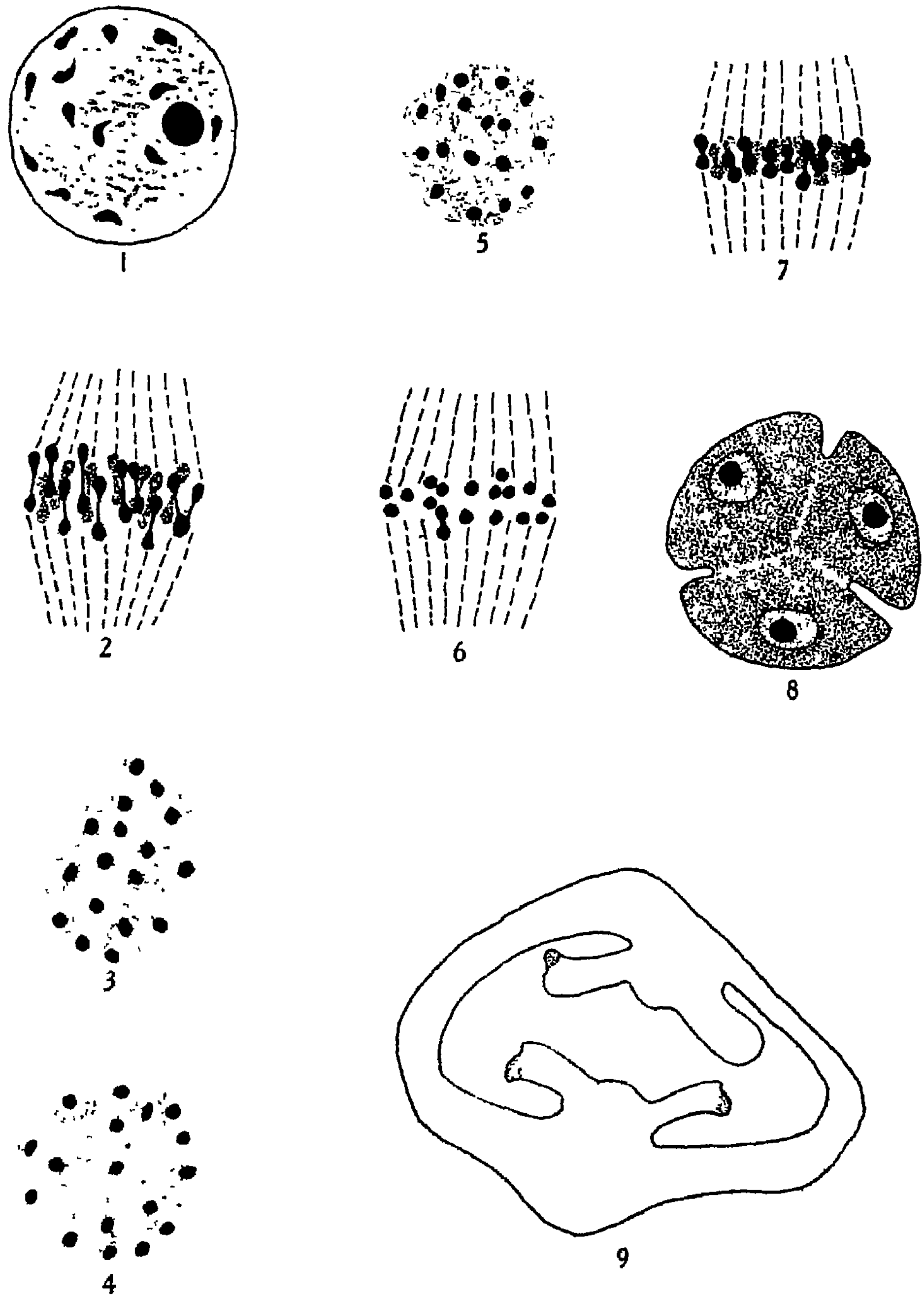
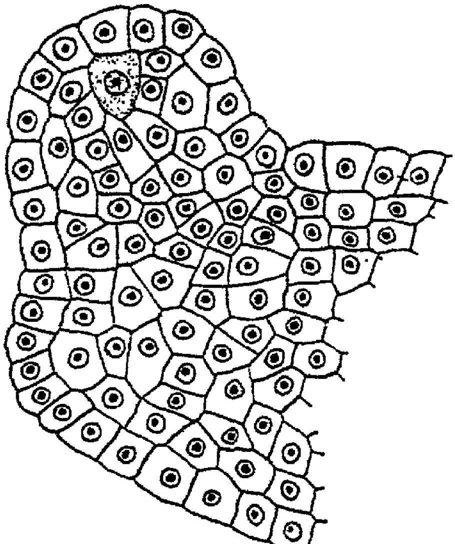


Fig. 1. Diakinesis. Fourteen of the univalents are shown. ×5700.
Fig. 2. Equational division in I. ×5700.
Fig. 3. Polar view of metaphase of I. 17 chromosomes. ×5700.
Fig. 4. Polar view of metaphase of II. 17 chromosomes. ×5700.
Fig. 5. Polar view of somatic metaphase. 17 chromosomes. ×5700.
Fig. 6. Endosperm mitosis in metaphase. 17 chromosomes. ×5700.
Fig. 7. Equational division in II. ×5700.
Fig. 8. Simultaneous microspore formation by furrowing. ×2400.
Fig. 9. Cross-section of ovary showing the four rows of ovules, the cylindrical funiculus and the young hemispherical nucellus (stippled). ×68.

is comparatively slight when that at the base is considered. After the few divisions have been accomplished the daughter cells become vacuolated and there is little or no further increase in number. The cells at the base of the nucellus, on the contrary, become meristematic, and by their divisions, longitudinal and transverse, enable the nucellus not only to elongate considerably but also to increase its basal diameter. In Fig. 11 are seen three stages in nucellar development, beginning with the archesporial cell stage (*a*) and ending at a stage taken at maturity of the embryosac (*c*). There is a further increase in size during the development of the endosperm. There is a conspicuous enlargement of the epidermal cells at the tip of the nucellus. They are at their maximum size by the time the embryo sac is mature (Fig. 26).

Development of the embryo sac

The single-celled archesporium first becomes distinguishable by increase in size of an axially located hypodermal cell. This occurs at a period in ovular development when the nucellus is but a small hemispherical group of cells, and the inner integument a slight protuberance when observed in a median longitudinal section through funiculus and nucellus (Fig. 10). Although the archesporial cell may be double the size of adjacent nucellus cells, its nucleus is scarcely larger than the other nuclei. The first division is periclinal, resulting in an outer parietal and an inner sporogenous cell (Fig. 12). The sporogenous cell at once elongates and at the same time greatly enlarges its nucleus. The primary parietal cell now initiates a series of periclinal divisions by which daughter cells are formed that overlie the sporogenous cell by a group from two to six cells in thickness, though the prevailing number, by far, is 3 (Figs. 14–19). Anticlinal divisions in the parietal cells may or may not accompany or follow the periclinal ones. If there are none, the parietal cells generally appear as a conspicuous axial row lying between the sporogenous cell and the epidermis at the tip of the nucellus (Figs. 18, 19, 21). Even with anticlinal walls present the boundaries of the parietal group may often be readily distinguished (Fig. 15); at other times with less certainty (Fig. 16). Schnarf (1929), also Coulter & Chamberlain (1903), state that division of the primary parietal cell occurs in a considerable number of species that have a wide distribution through the plant families. According to Schnarf (1929), the number of parietal cells produced varies generally between two and four. Periclinal divisions in the nucellus near its tip, accompanying those



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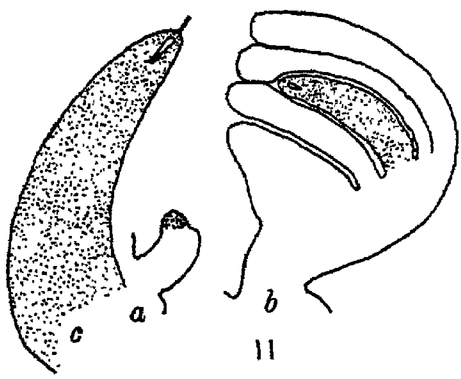

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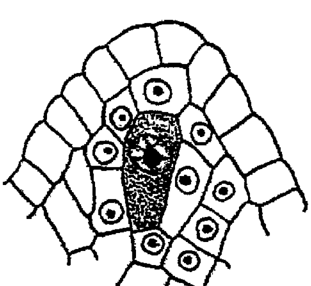

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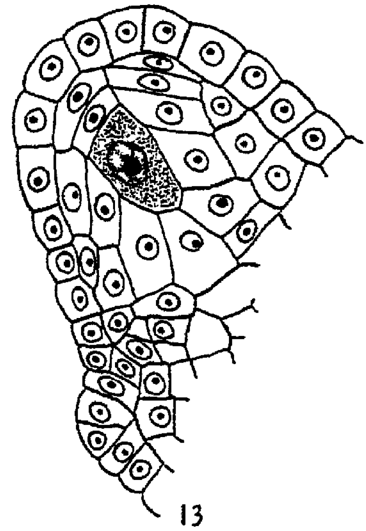

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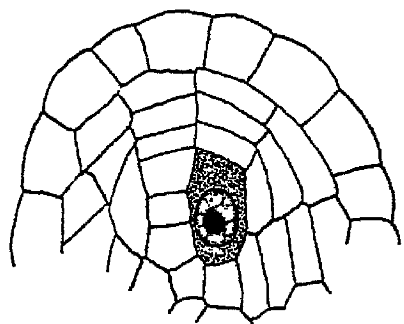

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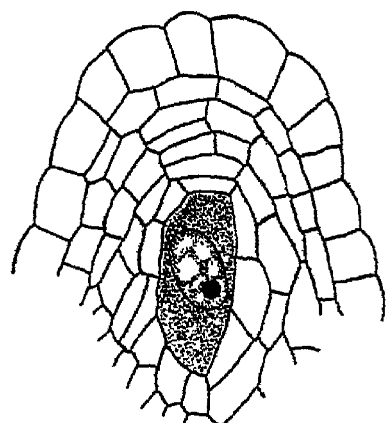

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Fig. 10. Young nucellus showing archesporial cell (stippled). Slight protuberance at base of nucellus is inner integument. × 690.

Fig. 11. Three stages in the development of nucellus (stippled). (*a*) at archesporial stage; (*b*) at time of first division of the embryo sac mother cell; (*c*) at the time of maturity of the embryo sac. × 75.

Fig. 12. Tip of nucellus showing primary parietal cell and sporogenous cell (stippled). × 800.

Fig. 13. Tip of nucellus showing the sporogenous cell (stippled) and two overlying cells which are produced by a periclinal division in the primary parietal cell. × 800.

Fig. 14. Tip of nucellus showing regular periclinal divisions in both parietal and nucellus cells. × 800.

Fig. 15. Tip of nucellus showing sporogenous cell and an overlying group of parietal cells with anticlinal walls. The boundaries of the group are fairly well defined. × 690.

of the parietal cell, are responsible for the apical elongation of the nucellus.

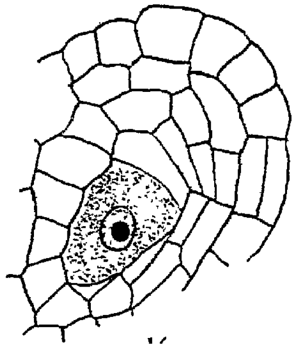
An examination of much material in the sporogenous cell stage, and following, plainly indicates that the cell typically develops directly into the embryo sac without the formation of megaspores. A single instance was observed, however, in which they were present, and their position and appearance were quite in accord with what would have been expected in such an instance. The usual three parietal cells, axially located and similar to those in other ovules, were present. Beneath them were three cells in the process of dissolution. Their blackened appearance gave indication that they were non-functional megaspores (Fig. 17). The two lowermost did not clearly show a separating wall, if any, due to their disintegrated state, but the two nuclei were still plainly discernible. Additional reasons for believing that the three cells lying directly above the embryo sac mother cell in this instance are megaspores rather than parietal cells, also that megaspores are not generally produced, are (1) parietal cells are not subject to dissolution till a much later stage in ovular development; (2) the nucleus of the embryo sac mother cell was found to be smaller in the functional megaspore than are nuclei in sporogenous cells that develop directly into embryo sacs; (3) binucleate embryo sacs typically lie in contact with the parietal cell directly above them without the presence of any intervening disintegrated cell substance (Fig. 21).

Schnarf (1929) recognizes six types of development from the archesporial cell stage to that of the mother cell. In his type III, a single archesporial cell differentiates and then divides to form an outer parietal and an inner sporogenous cell. The parietal cell undergoes further divisions, while the sporogenous cell becomes the embryo sac mother cell. It is clear therefore that the usual course of development in *I. arborea* follows type III.

The spindle of the first division of the embryo sac mother cell nucleus is longitudinal (Fig. 19). The stage shown is a late anaphase. The chromosomes are very small, approximately spherical and sufficiently well spaced at one of the poles to permit a count (Fig. 20). The number is 17, or the same that is found in nuclear divisions in the pollen mother cells, in the endosperm and in the somatic cells.

Vacuolation may have occurred before the first division in the embryo sac mother cell takes place (Fig. 18), but it is always a feature of the binucleate and trinucleate stages.

The nuclei of the binucleate embryo sac separate and take positions such that one lies over the other with a vacuole between. The lower nucleus increases in size and enlarges its nucleolus (Fig. 21). There



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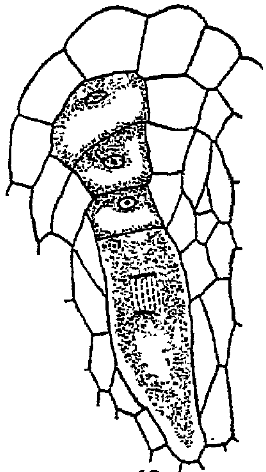

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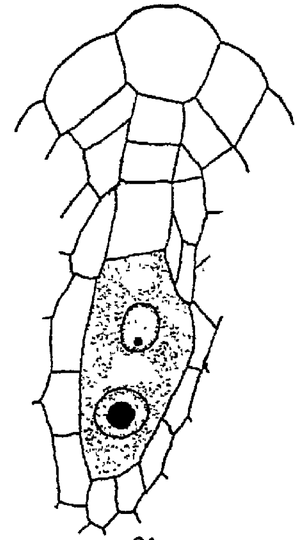

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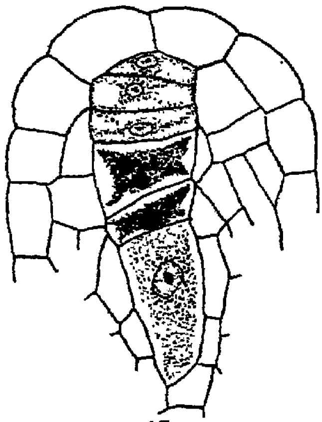

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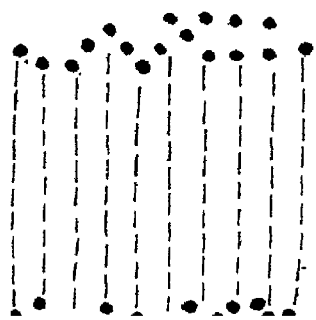

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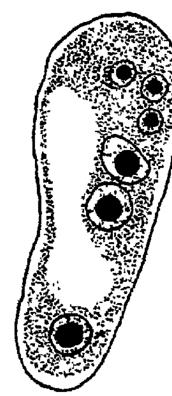

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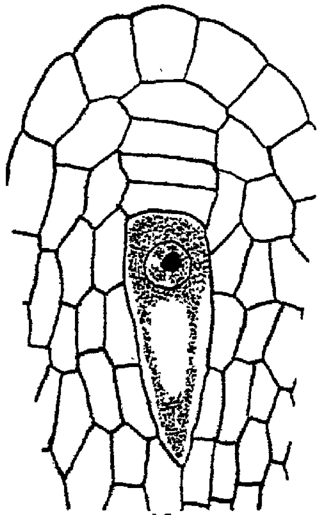

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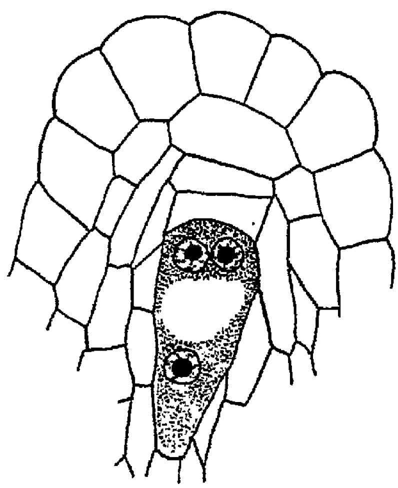

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Fig. 16. Tip of nucellus showing sporogenous cell and a parietal group with poorly defined boundaries. × 800.

Fig. 17. Tip of nucellus showing three parietal cells, three disintegrating megaspores and the functional megaspore. × 690.

Fig. 18. Tip of nucellus showing four axial parietal cells and a vacuolated sporogenous cell. × 800.

Fig. 19. First division of the embryo sac mother cell. × 690.

Fig. 20. Anaphase of first division in embryo sac mother cell. 17 chromosomes are discernible at one of the poles. × 5700.

Fig. 21. Binucleate embryo sac containing well-differentiated nuclei. × 690.

Fig. 22. Three-nucleate embryo sac. The nuclei are nearly the same size and show no organization. × 800.

Fig. 23. A typical 6-celled embryo sac in which a group of three lower are much larger than the three upper. × 690.

is, however, some variation in the time required for growth, and in the ultimate size of the nucleus and nucleolus. The nucleus nearer the micropyle undergoes a division, and while the spindle was not observed, the position of the daughter nuclei indicates that it is approximately transverse to the long axis of the embryo sac (Fig. 22). The three nuclei constitute the typical number for embryo sacs of the species. In one instance an exception was clearly evident, for six nuclei were observed (Fig. 23). They were in two groups of three each, the upper smaller, the lower larger. In a typical 3-nucleate embryo sac an upper group of two eventually consists of smaller nuclei than a lower single nucleus; hence it is tempting to suppose that the three lower and larger nuclei of the 6-nucleate sac are the progeny of the lower and larger nucleus in the binucleate stage, leaving the upper three as the daughters of the upper and smaller nucleus. In the few instances where more than three nuclei have been seen there has been no organization such as that found in a definitive embryo sac.

The typical definitive embryo sac in *I. arborea* has three nuclei which are organized into what appears to be an egg apparatus (Figs. 24, 25). The two upper, of pyriform shape with their associated cytoplasm, resemble synergids and are doubtless truly such. The lower nucleus may appear like an egg but perhaps more like an endosperm nucleus. There is a complete absence of polar nuclei and antipodals. The size of the embryo sac at this stage is relatively small when compared with the entire ovule as seen in median longitudinal section (Fig. 26).

Schnarf (1929) gives an excellent summary of the types of embryo sacs as outlined by Chiarugi (1927) and Rutgers (1923). None of the types listed, however, is identical with that exhibited by *I. arborea*. The *Plumbagella* type, which was first described by Dahlgren (1915), resembles that of *Isomeris arborea* in that a single archesporial cell divides to form a parietal cell and a sporogenous cell, the sporogenous cell typically becoming the equivalent of a megaspore in that it develops directly into an embryo sac without the usual formation of megaspores. The definitive embryo sac of *Plumbagella*, however, contains four nuclei as the typical number. Of these four, one becomes the egg nucleus, two become the polars, while the fourth is an antipodal. The polars fuse to form the primary endosperm nucleus. As meiosis is said to occur in *Plumbagella*, the egg nucleus would be haploid and the primary endosperm nucleus diploid. With no meiosis in *Isomeris arborea*, the chromosome complement of the three nuclei

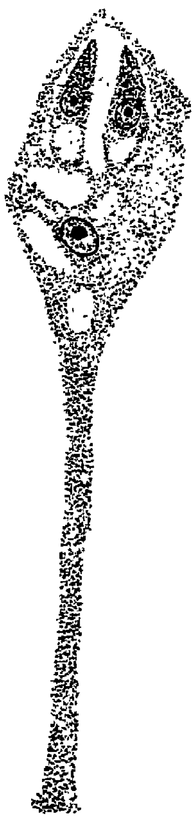
of the definitive embryo sac would be identical. There is a true egg in *Plumbagella* which develops into an embryo after being fertilized. In *Isomeris arborea*, what appears to be an egg is not truly such, since endosperm is the immediate tissue that originates from it and its surrounding cytoplasm. The embryo sac of this species doubtless belongs to an entirely new type, as it does not find a parallel in any listed by Schnarf (1929) nor in the literature issued since the publication of his book.

In the majority of ovules examined, a pollen tube was found to be present. After passing through the micropyle, the end of the tube enlarges to form a globose terminus, in which the tube nucleus may be readily seen. A short distance behind, in the narrow part of the tube, the sperm nuclei may be observed in favourable sections. Instead of discharging its contents into the embryo sac the bulbous end of the pollen tube develops a thickened wall and remains visible and a somewhat prominent feature, resistant to the disintegrating action of the growing endosperm, till this tissue is well along in its development (Fig. 27). As there is no discharge of sperm nuclei there is no fertilization, but the question arises, is the presence of the pollen tube a necessary stimulus to endosperm development? In an absolute sense the answer would have to be no, for embryo sacs have been found with endosperm in which there was no vestige of a pollen tube; while others have been found in which all three nuclei were intact, the ovule partially shrivelled, yet a prominent pollen tube terminus was seen in the micropylar end of the sac. It is generally true, however, that the majority of embryo sacs with endosperm also contain pollen tube termini.

BEHAVIOUR OF THE SYNERGIDS

The synergids generally disintegrate soon after endosperm development begins, but occasionally one of a pair becomes richer in protoplasm, enlarges its nucleus and undergoes a series of cell divisions. The separating walls are transverse to the long axis of the micropylar end of the embryo sac in which the synergid lies, so that a single row of cells is formed (Fig. 28). The number of cells produced is not constant, but the chain represented in Fig. 28 is an average of those attaining the longer length. An entirely different type of structure is seen in Fig. 29 in which the synergid develops a globose growth containing free nuclei. This was observed only once.

The stimulus that leads to synergid activity is unknown. It does not seem to be related to the presence of a pollen tube, for many



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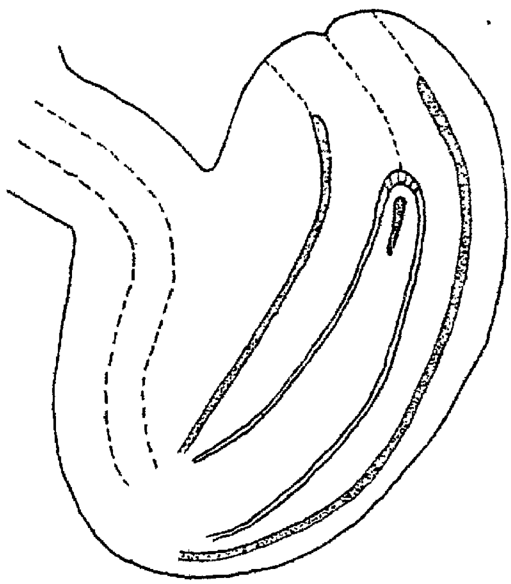

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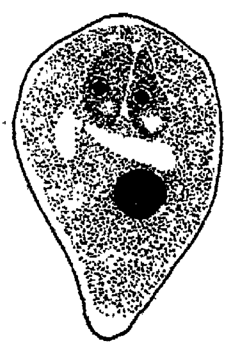

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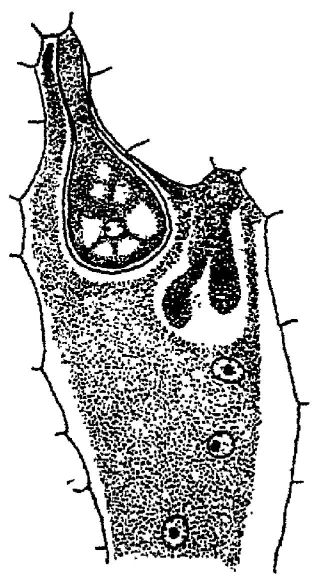

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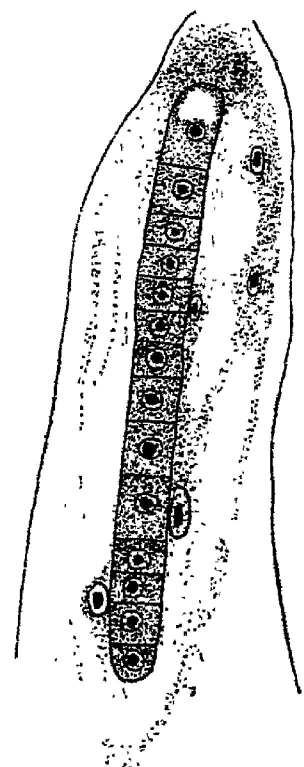

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Fig. 24. Mature embryo sac with two synergids and an endosperm nucleus. The lower portion of the sac has penetrated well into the nucellus. ×800.

Fig. 25. Unusually short example of mature embryo sac. The endosperm nucleus appears to consist entirely of nucleolus. ×800.

Fig. 26. Longitudinal diagram of ovule at the time of maturity of the embryo sac, showing its position in the nucellus and its relatively small size. Embryo sac, and cell layer of inner integument that develops sclereids, stippled. ×90.

Fig. 27. Micropylar portion of embryo sac showing bulbous terminus of pollen tube with its contained tube nucleus. A sperm nucleus is seen in the narrow part of the tube. Disintegrating synergids and young endosperm in the embryo sac. ×650.

Fig. 28. Chain of cells that have developed from a synergid. The free nuclei are those of endosperm. ×365.

embryo sacs containing pollen tubes show no synergid activity. No structure arising from a synergid has anything to do with endosperm or embryo formation for the following reasons: (1) it is absent from the majority of embryo sacs in which endosperm is developing normally; (2) endosperm in the free cell state has been found simultaneously with all stages of synergid activity; (3) the greatest length of any cell row falls far short of the position in which embryo development begins. There appears to be no purpose which these cell chains serve. They may be placed in the same class as active antipodals which function in some instances, at least, as nutriment to an encroaching endosperm.

THE SCLEREID LAYER OF THE INTEGUMENT

A layer of enlarged cells on the outside of the inner integument is evident in the ovule by the time endosperm formation begins. The transformation of the integument cells into the conspicuous, heavy-walled and pitted sclereids commences at the micropylar end and continues gradually down the ovule (Fig. 30). A layer corresponding to this has been reported by Guignard (1893) as occurring in *Polanisia* and *Cleome*.

THE ENDOSPERM

The first stage in development beyond the 3-nucleate mature embryo sac is the formation of endosperm from the nucleus that lies near the synergids. Considerable interest and importance attaches to this nucleus. In general appearance and position it more nearly resembles a primary endosperm nucleus than an egg, since an egg would lie typically in closer relationship with the synergids, forming a so-called "egg apparatus". While endosperm is the first tissue formed, it should be realized, as will be described in full later, that this nucleus with its cytoplasm is the ancestral cell of the embryo. Although no embryo sac exactly similar to that in *Isomeris arborea* has been described, so far as the author has been able to discover, that in *Plumbagella* as described by Dahlgren (1915) resembles it in the respect that two nuclear divisions occur between the mother cell and the mature embryo sac. In *Isomeris arborea*, however, one of the daughter nuclei of the first division does not undergo a division previous to the short resting period which appears to characterize the mature embryo sac. Further comparison with *Plumbagella* results in complete diversity. The *Plumbagella* embryo sac contains an egg, two polars and a single antipodal—four nuclei in all, while

that in *Isomeris arborea* contains two synergids and a primary endosperm nucleus—three nuclei in all. There is a primary endosperm nucleus finally in *Plumbagella*, but it is the result of nuclear fusion. The endosperm nucleus in *Isomeris arborea* is single in origin and is haploid with respect to its chromosome complement, as was definitely determined by a count in one of its dividing daughter nuclei (Fig. 6).

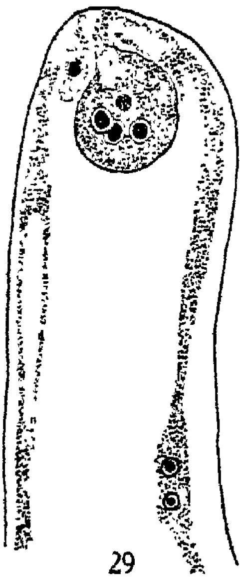
There have been a few cases recorded in which endosperm has arisen from the upper polar alone, that is, without fertilization or union with the other polar. Examples of this have been found in *Balanophora elongata* and *B. globosa* by Treub (1898), Lotsy (1899) and Ernst (1914). In these two species there is no reduction division, the evidence pointing to a diploid condition through their entire life history.

Chiarugi (1927) has applied the term *oangium* to the group of nuclei that typically occupy the micropylar end of the embryo sac. The term may include egg, synergids and one free nucleus; but while certain of these may be absent, the egg is an essential constituent. The term is therefore inapplicable to the group of three nuclei in *Isomeris arborea*. No designation is available, but on the basis of naming the group after its most important nucleus, the term *endangium* would not be inappropriate.

Free-cell formation is the first step in endosperm development. Some of the daughter nuclei move up into the micropylar tip of the embryo sac while others pass down along its inside wall where with the connecting cytoplasm they form a thin and closely appressed layer. In the meantime the size of the embryo sac increases greatly, especially in length, and as the ovule is of the campylotropous type, the embryo sac becomes J-shaped, a form which was noted in *Polanisia graveolens* by Guignard (1893).

As endosperm develops further in the free-celled state, a second and different type of endosperm arises from it which is here designated as nodular. This begins by a darkening of the cytoplasm in the vicinity of nuclei, accompanied by the formation of coarse granules which stain black in the presence of iron-alum haematoxylin (Figs. 31, 32). Soon there is a tendency for these dark areas to extend and fuse into cylindrical or even globose masses which become more coarsely granular (Fig. 33). At first no walls surround or divide the nodules (Fig. 31), but their later formation separates the nodules from surrounding cytoplasm and the free nuclei (Figs. 32, 33).

Distribution of endosperm nodule formation varies somewhat in the different ovules. In many instances it is chiefly in the lower or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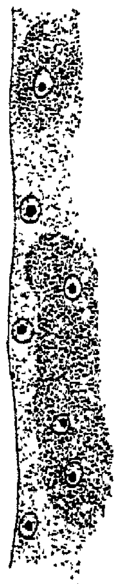
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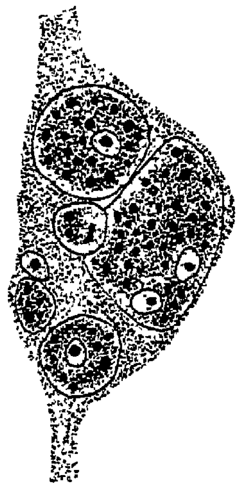
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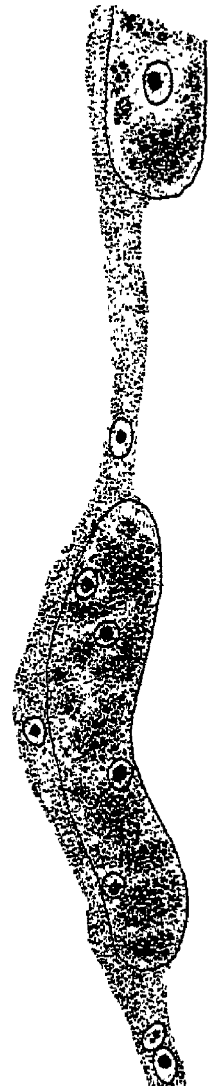
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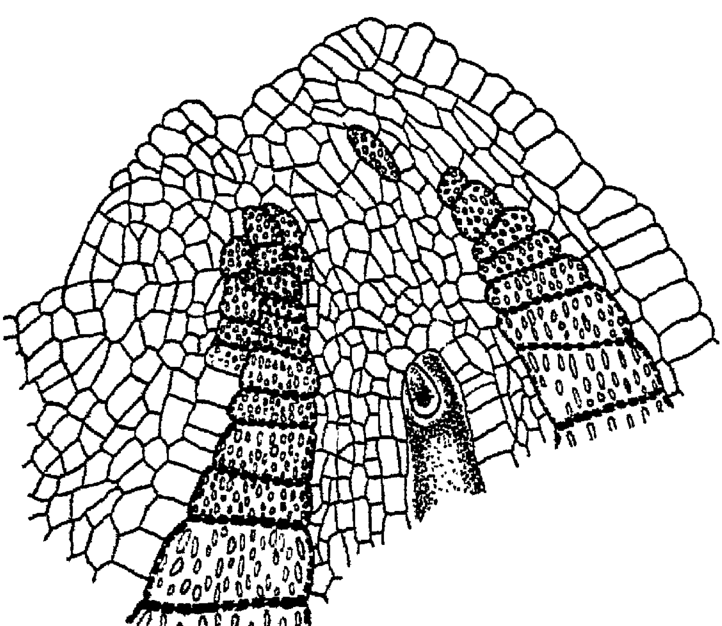
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Fig. 29. Globose structure containing free nuclei, which has developed from one of the synergids. ×365.

Fig. 30. Tip of ovule showing the sclereid layer of the inner integument. ×200.

Fig. 31. Early stage in nodular endosperm formation. ×365.

Fig. 32. Endosperm nodules showing beginning of wall formation. ×365.

Fig. 33. Endosperm nodule showing late stage in wall formation. ×365.

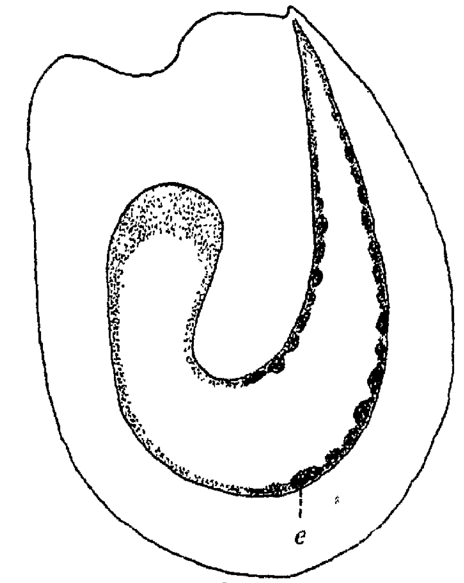
curved portion of the embryo sac (Fig. 34). It is not difficult, however, to find nodules in the micropylar region and extending along the inside wall of the sac to the beginning of the short arm of the J. There is variation also in the quantity of endosperm given over to nodule formation. An instance in which it is abundant is represented in Fig. 35. This drawing was made from a section which was parallel to one side of the embryo sac and which was cut lengthwise through its endosperm lining—quite a different appearance from that of a median section. It is evident that the nodules may be bent and occupy the larger space proportion of the lining endosperm. As nodule formation does not extend into the short arm or chalazal portion of the embryo sac, a considerable mass of cytoplasm and free nuclei are found in it during the earlier period of endosperm development. An interesting feature of these free nuclei is that they are sometimes seen in identical mitotic stages, for example, metaphase.

The embryo sac at the age represented in Fig. 30 contains only a small proportion of endosperm when its entire content is considered, most of the embryo sac being filled with fluid. The endosperm at this stage consists of two kinds, nodular and free-celled. The nodular contains free nuclei, but these are in small groups enclosed within darkly staining, granular cytoplasm which is enclosed by a wall. A third kind of endosperm arises from the extra-nodular free nuclei and their surrounding cytoplasm. This is a cellular type which is in marked contrast in appearance to the other two. The first step in its organization is a vacuolation of the cytoplasm lying between the free nuclei. The nuclei seem to be set off in cells but the intervening walls do not give a cellulose reaction and hence may be only cytoplasmic membranes. Cell division begins, the tissue thus formed extending inwards and encroaching upon the central cavity. Mitosis in these endosperm cells begins with a narrow spindle which, however, soon broadens greatly at the equator till the side of the cell is reached. In the meantime the daughter nuclei have reached the resting stage. An optical section through a late stage shows but a few fibres attached near the centre of the cell plate (Fig. 36). A surface view of the spindle seen in this late stage shows that the peripheral fibres are numerous and close together. These persist for some time after the cell plate has reached its greatest diameter, after which they gradually disappear. As the cellular type of endosperm increases many of the nodular masses become enveloped by it (Fig. 37).

ORIGIN AND DEVELOPMENT OF THE EMBRYO

During the earlier part of the investigation of *Isomeris arborea*, an embryo was sought in the micropylar end of the embryo sac. The almost invariable absence of one in this region was at first accounted for by a supposed development of a long suspensor that would push the embryo proper well down toward the middle of the embryo sac. The extremely evanescent property of the suspensor in certain species has doubtless led some investigators to report embryos having endosperm origin. Attention has been called to the absence of a distinctive egg in *I. arborea*. The only remaining structure in the micropylar region from which an embryo could be derived would be one of the synergids which occasionally, though not generally, forms a cell row resembling a suspensor. It has already been explained, however, that such a cell row falls far short of elongating to the point where the embryo generally takes its origin. A search for young embryos in ovules with only a moderate amount of endosperm has always yielded negative results. Only after the ovule has reached a comparatively large size, and nodule formation has about reached completion, has the presence of an embryo been noted. By an almost invariable rule the embryo arises near the bottom of the long arm of the embryo sac, a position which is about equidistant from the micropylar and chalazal ends (Fig. 34 *e*). At no time was an embryo found within the short arm of the embryo sac. Three positions of embryos are represented in Fig. 38. As stated above, by far the most common location is the lowest one, or that near the concave bend of the embryo sac. Only a few have been noted near the convex bend, and only once was one found near the micropylar region.

In all young embryos observed there was found to be a very direct connexion between them and the nodular type of endosperm. As all endosperm consists of only a thin lining layer at the time embryos begin to form, their position is at first peripheral. As there is apparently no egg, or single recognizable cell from which the embryo arises, the first indication that embryo formation is about to begin is best observed by following through a series of sections, with attention directed to nodules located at the bend of the embryo sac where, as already indicated, they are most likely to develop (Fig. 38 *e*). The first noticeable sign is the extension of the chalazal end of some endosperm nodule into a blunt point (Fig. 39). There is undoubtedly a division of the nuclei in the vicinity of this point till a small group



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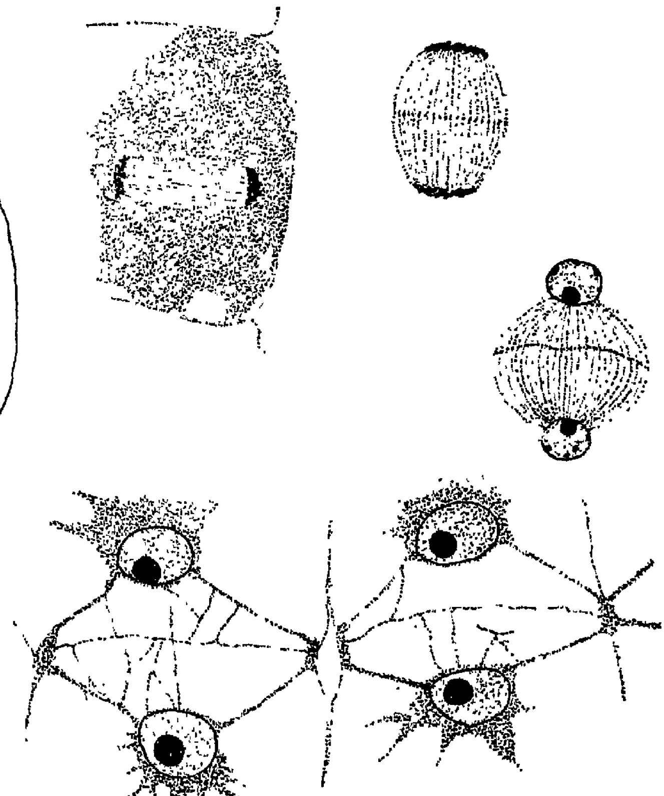

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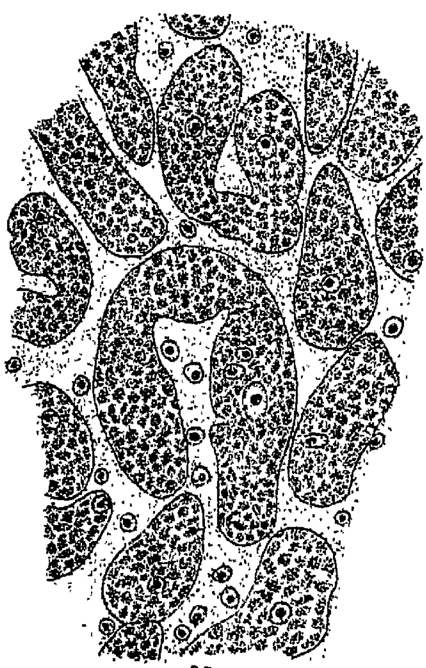

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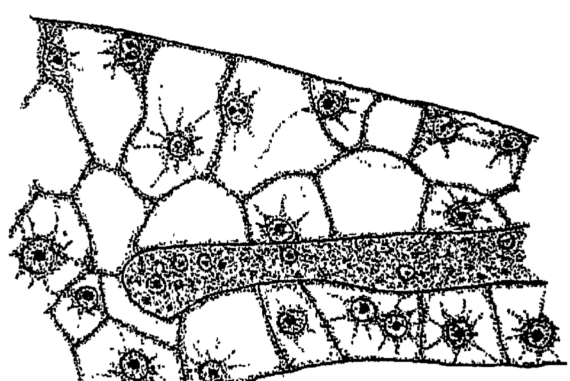

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Fig. 34. Diagram of ovule at about the time embryo formation begins. The usual position of the nodule that originates the embryo is indicated at (*e*).
Fig. 35. Endosperm nodules as seen in a section parallel to one side of the embryo sac. × 365.
Fig. 36. Endosperm mitoses showing the development of the cell plates. × 800.
Fig. 37. Cellular type of endosperm which has completely enclosed a nodule. × 200.

is formed which, in some instances at least, range smaller in size than those nearer the micropylar end of the nodule. The blunt point soon extends itself into a cylindrical process into which some of the adjacent nuclei migrate (Fig. 40). About this time there generally forms a series of curved walls across the nodule, which when seen in section present their concave surfaces toward the endosperm process mentioned above. The number of segments thus formed from the nodule varies somewhat, but they generally agree in being multinucleate. Approximately simultaneously with the formation of the curved walls in the nodules, cross walls originate within the nodular process and separate the contained nuclei into one- to several-nucleate cells (Figs. 41–44). The cylindrical process is now distinctly recognizable as a pro-embryo. While the first walls formed in the pro-embryo are at right angles to its long axis, longitudinal walls appear very early, but the order of their formation is not constant. Further development results in the differentiation of the proximal portion of the pro-embryo (that next to the nodule) into a suspensor, and of the distal part into the embryo proper. The terminal hemispherical cell undergoes two divisions, accompanied by longitudinal walls, to form the quadrant stage (Fig. 45 *a–c*). It is not certain, however, whether the terminal cell is always from the first uninucleate. Many embryos show two nuclei in this cell, with no separating walls, so that it is possible that more than one nucleus was allotted to it before the basal cross wall was formed. As many as four nuclei have been seen in this cell with no discernible separating walls (Fig. 42). The octant stage and then the cutting off of the dermatogen occur in successive order (Fig. 46). There is no single cell which acts the part of a hypophysis.

Suspensor cells may be uninucleate or, for a time at least, multinucleate. As there are always some longitudinal walls formed, the cells are not entirely uniseriate. Suspensors vary in length, wide differences being noted in Figs. 47–50. The cause of this variation is doubtless the difference in length of the pro-embryos, extremes being seen in those represented in Fig. 45 *b* and *d*. The suspensor, hence the underlying developing radicle, is always directed toward the micropylar end of the embryo sac. It is difficult to explain this remarkable polarity in the absence of a directing suspensor, or any definite connexion with either end of the embryo sac. While suspensors have been noted on all embryos observed, their usual function, that of pushing the developing embryo into a more nearly central and thus favourable position respecting nutrition, is scarcely applicable here,

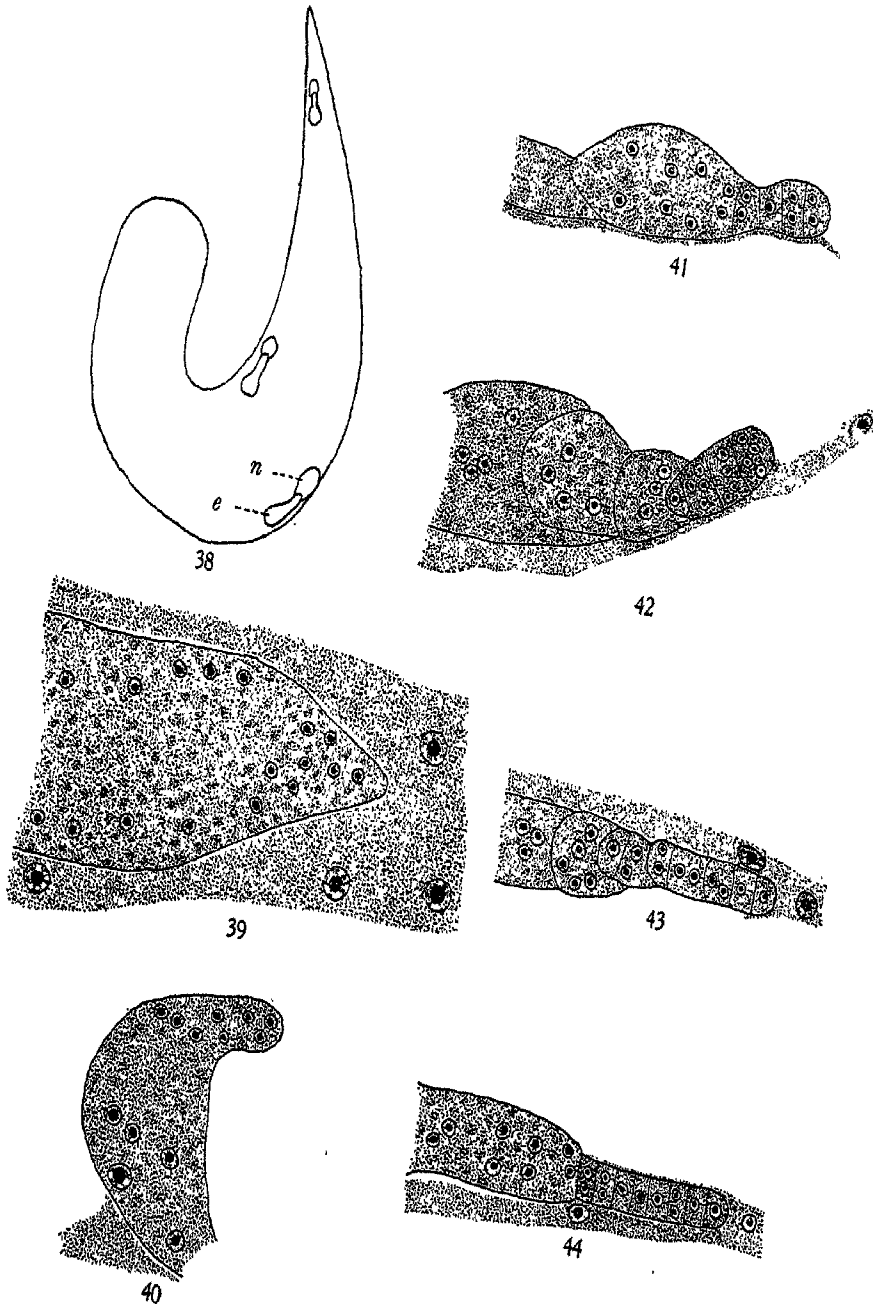
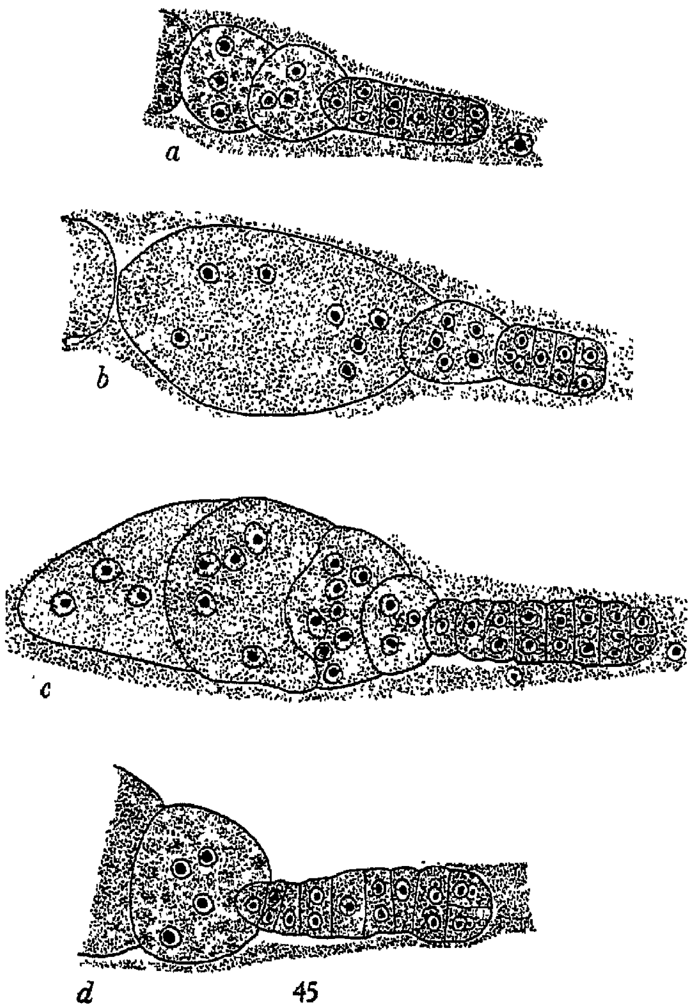


Fig. 38. Diagram of embryo sac in longitudinal section showing three different locations of embryo development. *e*, embryo; *n*, endosperm nodule from which the embryo is derived.
Fig. 39. Endosperm nodule with pointed end which is an early stage in embryo formation. $\times 365$.
Fig. 40. Endosperm nodule developing a pro-embryo by means of a cylindrical, multinucleate process. $\times 365$.
Fig. 41. Young embryo with early cross walls. Concave wall formation within the nodule has not been completed. $\times 365$.
Fig. 42. Young embryo in which longitudinal wall formation is comparatively late. $\times 365$.
Fig. 43. Young embryo in which the development of cross walls is irregular. $\times 365$.
Fig. 44. Young embryo with attached endosperm nodule within which concave wall formation has not begun. $\times 365$.

since the embryo is from the first apparently in as favourable a position as possible, being in the centre of endosperm development. The connexion between the suspensor and the endosperm nodule



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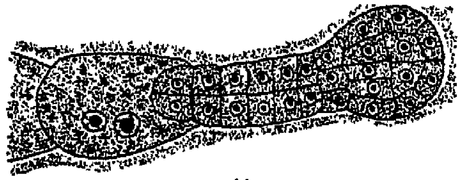

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Fig. 45. Stages in quadrant formation, with variation in embryo length and in number of concave walls in endosperm nodules. × 365.
Fig. 46. Formation of dermatogen in the octant stage. × 365.

from which it took its origin is, however, for a considerable time maintained, and it is possible that some nutriment passes from the nodule through the suspensor to the embryo.

A half-grown embryo with well-developed cotyledons is shown in Fig. 51. It is seen to lie horizontally in the curved base of the J-shaped embryo sac, its radicle directed toward the micropylar canal, its cotyledon tips toward the chalaza. Additional growth in length is accompanied by further bending till maturity is reached,

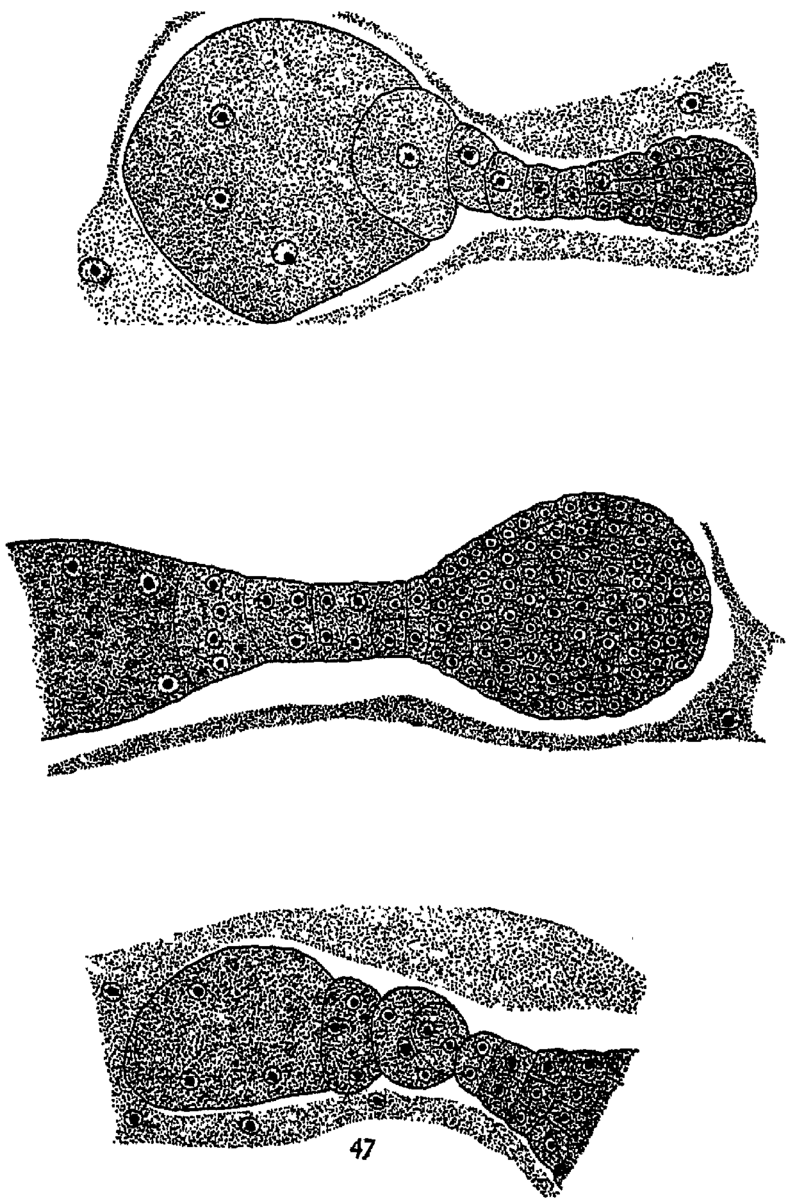


Fig. 47. Older embryos showing variation in suspensors and endosperm nodules. ×365.

when the embryo takes the coiled form seen in the mature seed. Only 10–12 % of the ovules ripen into seeds. In a single ovary between 40 and 50 ovules start development, necessitating a close placement. As mature seeds are large, the increase in size of ovules soon results in crowding, so that a majority must give place to others. Since all

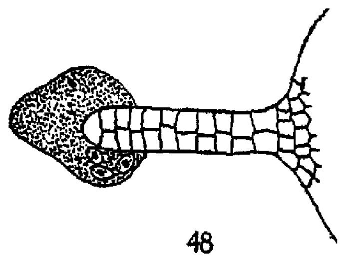
ovules grow to some degree after pollination it would appear that competition for space is responsible for the large percentage of loss. Only five or six seeds on an average are found in the mature capsules.

Instances of endospermous origin of embryos have been reported from time to time, though they are apparently rare. Thus far, however, no case has been cited, to the author's knowledge, in which a single cell plays a double role without the presence of a true egg. Rosenberg (1908) reports an instance of embryo origin from endosperm in *Hieracium excellens*, in the embryo sac of which two embryos may arise, one typically by the fertilization of an egg, the other adventitiously from endosperm. Rosenberg (1908) suggests, however, the possibility that the adventitious embryo may have arisen from a polar nucleus that had failed to fuse with the other. Other species of *Hieracium* are occasionally cited as examples of apogamy due to a diploid egg. Schnarf (1919), for example, finds that *H. aurantiacum* is an apogamous species in which two embryos may arise within the same embryo sac, one being derived from a diploid egg, the other probably from endosperm. He states that he hoped to find better material later with a view to definitely establishing the endosperm origin of one of the embryos. *Balanophora,* formerly thought to have been an example of embryo origin from endosperm, has been shown by Ernst (1914) not to be such. The situation in *Hieracium,* as can be readily understood, is not an exact parallel of that in *Isomeris.* The nearest approach, perhaps, is that found in *Hieracium excellens* in which a haploid egg exists, along with the possibility that the embryo may have arisen from an unfused polar. Thus the embryo and endosperm would be haploid as in *Isomeris arborea.*

The literature on the subject of endosperm origin of embryos leaves one in doubt as to any definite and well-attested instance, since the manner of development is not clearly stated. The stages in embryo development in *I. arborea* preclude any other derivation for it than that of endosperm.

THE NATURE OF THE ENDOSPERM AND EMBRYO

That the mature embryo sac of *I. arborea* is a female gametophyte is indicated by two facts: the pollen tube is directed into it, and the organization resembles that of a female gametophyte more closely than that of any other structure. The endosperm nucleus, one of the three found in the gametophyte, continues division, after a pause, without fusion with a sperm or with another polar. It undoubtedly



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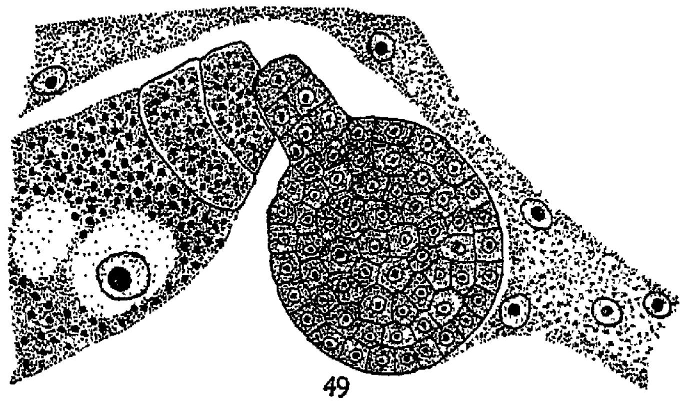

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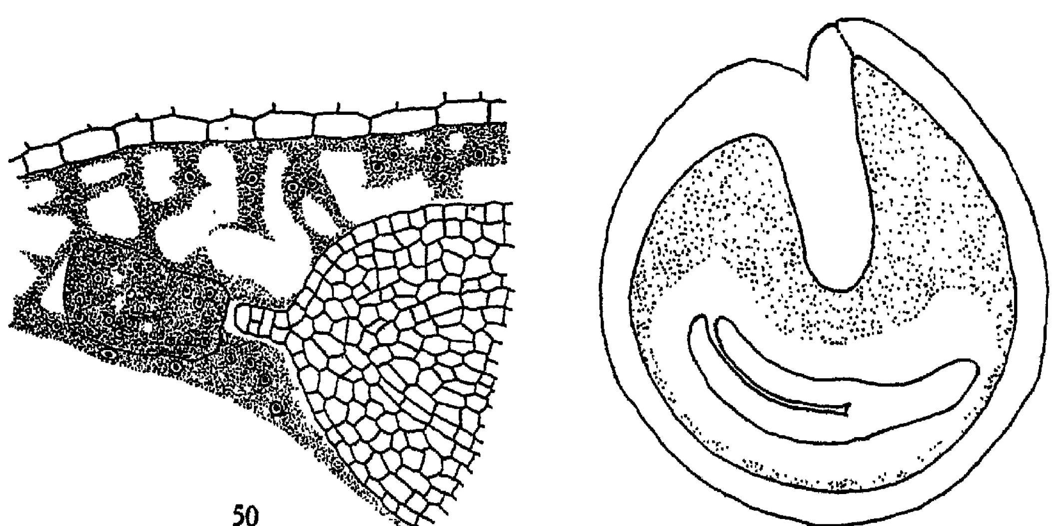

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Fig. 48. Portion of older embryo with unusually long suspensor, the tip of which is embedded in an endosperm nodule. × 200.

Fig. 49. Embryo with all cells uninucleate, including those of the suspensor. The angle formed by the long axes of the embryo and nodule is probably due to these structures being pushed out of alignment in manipulation. × 365.

Fig. 50. Radicle end of embryo in young cotyledon stage. An endosperm nodule is near the suspensor. × 90.

Fig. 51. Diagram of a section through a young seed which contains a nearly half-grown embryo. Endosperm stippled.

develops endosperm without any alteration in its nature. As it is gametophytic when a constituent element of the female gametophyte, its descendant nuclei and tissue are likewise gametophytic. In fact, according to Coulter (1911), all endosperm is gametophytic because it continues the tissue of the gametophyte. The following paragraphs from his paper on "The Endosperm of Angiosperms" (1911) constitute a portion of his conclusions:

(1) Endosperm formation is not dependent upon the presence of a male nucleus.

(2) Endosperm formation is not even dependent upon a polar fusion.

(3) Therefore both these fusions may be regarded as supplementary rather than determinative.

(4) Endosperm formation does not even depend upon having been preceded by reduction division.

(5) The fusions associated with endosperm formation do not represent a definite process, but are miscellaneous in number and order.

(6) The product of such fusions as do occur is merely an undifferentiated tissue which practically continues the tissue of the gametophyte, that is, it is simply growth and not organization.

Paragraphs (1), (2) and (4) apply to the origin of the endosperm in *I. arborea*, and it would appear that only very rarely are all three conditions found in a single species. Continuing in his conclusions Coulter (1911) says: "The whole history of the megaspore and its products justifies us in regarding this tissue [endosperm], however formed, as gametophytic." In another place he states: "It is pertinent to ask, therefore, why there should be any hesitation in recognizing the endosperm as gametophytic from its lack of organization, no matter how it originates. It is obvious that the constancy of endosperm lies in its structure and not in its origin."

It would seem at first thought that the embryo in *I. arborea*, being in nuclear constitution similar to and a direct outgrowth from the endosperm, could be nothing else than gametophytic; but according to Coulter (1911), the embryo is a sporophyte whether it is the result of the act of fertilization or not, for it is recognized by its organization. In line with his conclusions we find that the endosperm of *I. arborea* is gametophytic because of its evident lack of organization; while the embryo is sporophytic because of the presence of organization, though on account of its haploid chromosome number the term haplodiplont is bestowed upon it. It is of interest, however,

to note that two structures in *I. arborea*, the endosperm and the embryo, of identical origin and chromosome content, one a direct outgrowth of the other, are regarded, one as a gametophyte and the other as a sporophyte.

SUMMARY

Isomeris arborea differs markedly in its megagametophyte, and in the origin and development of its pro-embryo, when compared with other investigated species of the Capparidaceae.

An anomaly appears during microsporogenesis in *I. arborea* in that there is no fertilization nor meiosis, with univalents only appearing in diakinesis. Chromosome counts were obtained in I and II, in the endosperm and in the somatic cells. All were 17. As the range of *n* counts in other investigated species of Capparidaceae is in the proximity of 17, it is probable that *I. arborea* is a haplodiplont, and is perhaps the first recorded, well-established wild species exhibiting monoploidy.

Formation of microspores is by the simultaneous method and by furrowing.

There is a single archesporial cell which is hypodermal in origin. Its first divisions result in a series of about four parietal cells that are usually axial in position. After their production megaspore formation does not generally follow, but when it does occur the non-functional megaspores soon disintegrate. The parietal cells become indistinguishable from nucellus cells and remain till the nucellus is absorbed. An embryo sac of three cells is formed, there being two synergids and an endosperm nucleus. No true egg is formed.

A pollen tube may enter the micropyle and form a bulbous terminus in the embryo sac. It does not discharge its contents, but instead develops a thickened wall and remains intact during the earlier stages of endosperm development.

The synergids generally disintegrate at the beginning of endosperm development, but one of a pair occasionally continues activity, becomes richly protoplasmic, and by cell division forms a cell row that extends a short distance downward from the micropylar end of the embryo sac. This activity seems to serve no important useful purpose.

Endosperm begins as free-cell formation but afterwards exhibits two definite types of structure, a nodular type consisting of circumscribed masses of coarsely granular cytoplasm containing free nuclei;

a cellular type consisting of vacuolated cells in the form of a tissue. The nodular type does not extend into the short arm of the J-shaped embryo sac.

The embryo arises by a direct outgrowth of an endosperm nodule which is generally located near the concave base of the long arm of the embryo sac, though it may originate from a nodule located near the base of the convex curve of the sac. In one instance an embryo was found developing from a nodule in the micropylar region.

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THE FORMATION OF OXALIC ACID BY *ASPERGILLUS NIGER*

By A. ALLSOPP
Department of Botany, University of Manchester

(With 12 figures in the text)

Introduction

Certain strains of *Aspergillus niger* produce large quantities of oxalic acid in the presence of a wide variety of organic substrates. The formation of oxalic acid by *A. niger* was first investigated by Wehmer (1891 *a*, *b*), but he made no attempt to explain the chemical nature of the changes involved. At the present day, despite the attention of many workers, there is still surprisingly little exact knowledge of the chemistry of the process.

Almost all research on the mechanism of the conversion of glucose into oxalic acid has been based on feeding experiments, in which it is assumed that if the substance under investigation is a member of the normal sequence of reactions it should yield oxalic acid at least as quickly as glucose. Failure to obtain oxalic acid is taken to indicate that the substance supplied plays no part as an intermediate product.

The most noteworthy work of this type was carried out by Raistrick & Clark (1919), who grew *A. niger* on culture solutions containing salts of various organic acids. After the lapse of about 5 weeks it was found that oxalic acid had been produced in quantity from the salts of malic, tartaric, succinic, fumaric, and acetic acids. The case of lactic acid was outstanding for, although growth was heavier than in any other instance, no oxalic acid was detected.

From the fact that oxalic acid was not obtained from the salts of the 3-carbon acids, Raistrick & Clark concluded that glucose degradation does not follow the Neuberg reactions in *Aspergillus*. They held the view that 1 molecule of glucose gives rise to 1 molecule each of oxalacetic acid and acetic acid. The oxalacetic acid is said to be hydrolysed into oxalic and acetic acid, and both molecules of acetic acid arising from the glucose are believed to undergo further oxidation to oxalic acid. This hypothesis, according to which oxalacetic acid is the immediate precursor of oxalic acid, has the advantage of

explaining why oxalic acid is produced from the 4-carbon but not from the 3-carbon acids.

The type of experiment on which these views were based is not free from objections. It is shown in the experimental part of this paper (cf. also Butkewitsch, 1934 *a, b*) that oxalic acid can arise in considerable quantity from the contents of the mycelium. Therefore, when solutions containing salts of organic acids as the sole source of carbon are inoculated with spores of the fungus, it is not possible to decide whether any oxalic acid present after several weeks of growth has arisen directly from the acid supplied, or from the mycelium produced at the expense of this acid. Raistrick & Clark were aware that formation of oxalic acid, when the fungus is grown on a given acid, does not necessarily imply that the acid is one of the intermediates, but assumed that "a failure to produce oxalic acid from the substance under investigation justifies the conclusion that the substance does not represent a stage in the reactions involved in the production of oxalic acid". Since their oxalate determinations were carried out only after about 5 weeks of growth any oxalic acid formed in the earlier stages might have disappeared before this time.

The fact that the salts of the acids were used is a further argument against the validity of their conclusions. Since growth of the mycelium proceeds at the expense of the organic anions, it is clear that, when the salt is used, the medium will become increasingly alkaline and will tend to trap any acids produced by the fungus. Thus, acid present in the culture medium may have arisen from the carbohydrate reserves of the mycelium as a result of the presence of hydroxyl ions generated by assimilation of the organic anions. When the salts of malic, succinic, tartaric, fumaric, and acetic acids are used as a source of carbon, the acid formed to neutralize the free base is known to be oxalic acid, but the nature of the acid produced when salts of pyruvic, glyceric, glycollic, glyoxylic, and formic acids are employed is still unknown.

The absence of oxalic acid in certain cases may be a result of the fact that some of these organic acids, especially lactic acid, markedly inhibit the production of oxalic acid from carbohydrate. Our results are described more fully in Group 4 of the experimental part of this paper, but it may be pointed out here that the effect is not due merely to the *p*H of the medium, since hydrochloric acid of the same *p*H had practically no inhibiting power. Apparently this inhibiting effect of certain acids was not realized by Raistrick & Clark, or other previous workers.

The objections discussed above apply equally to the work of most investigators of the acid metabolism of the mould fungi. There can be little doubt that the unsatisfactory nature of the experimental methods is responsible for the contradictory results and the divergent views of the different workers on oxalic acid formation.

In the majority of hypotheses of the mode of origin of oxalic acid, acetic acid is regarded as the forerunner of oxalic acid, but there is only one unconfirmed record of the production of acetic acid by *Aspergillus* (Heinze, 1903).

There are several diverse views relating to this supposed conversion of acetic acid into oxalic acid. Challenger *et al.* (1927) produced evidence of the direct oxidation of acetic to oxalic acid via glycollic and glyoxylic acids. From cultures grown on calcium acetate they obtained glyoxylic and glycollic acids, with calcium glycollate glyoxylic acid resulted, while ammonium glycollate yielded oxalic acid. But the acids detected might have arisen from carbohydrates within the mycelium and been trapped by free base resulting from the assimilation of the anions of the substrate. In addition, the amount of oxalic acid obtained was too small to justify the acceptance of this scheme as the normal course of sugar degradation.

The results of Bennet-Clark & La Touche (1935) are in opposition to the hypothesis of the origin of oxalic acid from acetic acid via glycollic and glyoxylic acids. They found that glycollic acid disappeared at a fairly high rate without any corresponding appearance of oxalic acid. Similar results were obtained with citric acid, suggesting that this acid also is not an intermediate product in oxalic acid formation.

An alternative view to the direct oxidation of acetic acid considers that the production of succinic acid by the Thunberg reaction is the first step. Bernhauer & Scheuer (1932) supported this view. They obtained no oxalic acid from sodium glycollate and large amounts from sodium succinate. But at a later date Bernhauer & Slanina (1934) obtained equal quantities from the sodium salts of the two acids. The latter result suggests that the oxalic acid was trapped in neutralizing the free sodium ions liberated from the salts by the utilization of the organic anions.

The exact nature of the conversion of succinic to oxalic acid is interpreted differently by different workers. Bennet-Clark (1933) suggested the following scheme:

Succinic acid $-2H \rightarrow$ fumaric acid $\rightarrow$ oxalacetic acid $\rightarrow$ oxalic + acetic acid.

This hypothesis of the "acid hydrolysis" of oxalacetic acid was not confirmed by feeding the fungus with a solution of oxalacetic acid.

Experiments described in the experimental section of this paper lend no support to the view that oxalacetic acid is the precursor of oxalic acid.

Recently, attention has been turned to the possibility of oxalic acid arising from formic acid. Chrząszcz & Zakomorny (1933) and Bernhauer & Slanina (1933, 1934) obtained oxalic acid when cultures were reared on solutions of formates. These results were adduced as evidence of the formation of oxalic acid by the dehydrogenation of formic acid. But as is also true for much of the work on oxalic acid formation, there is no certainty that the results are not due purely to the fixing of oxalic acid formed from carbohydrate, by the free base left after the absorption and assimilation of formic acid.

Bernhauer & Slanina (1934) also suggested that the tribasic acids afford a possible source of oxalic acid. Their cultures reared on the sodium salts of aconitic and citric acids yielded practically the same amount of oxalic acid as cultures supplied with acetates. But these results are precisely those to be expected if oxalic acid is produced from the carbohydrate reserves of the mycelium and fixed by the free base made available by the assimilation of the anions of the salts. Neglecting this possibility, Bernhauer & Slanina explained their results by the following scheme:

$$\text{Citric acid} \rightleftharpoons \text{aconitic acid} \rightarrow \begin{cases} \text{glyoxylic acid} \rightarrow \text{oxalic acid} \\ \qquad\qquad\qquad\quad + \\ \text{oxalacetic acid} \rightarrow \text{oxalic acid} \ + 2CO_2. \end{cases}$$

It is possible that some of the reactions postulated by these and other workers may occur in fungus mycelia supplied with the salts of organic acids; but it seems unlikely that such reactions play any part in the normal conversion of glucose (or reserve carbohydrate) to oxalic acid in the absence of an alkali, which would act as a trap.

The view advanced in this paper is based on the facts (dealt with in detail in the experimental section) that if fed to the fungus in the absence of an alkali trap, none of the 4-, 3-, or 2-carbon acids referred to in the various hypotheses discussed above will yield oxalic acid. Glucose, certain other hexoses and pentoses, and gluconic acid, on the other hand, give large yields of oxalic acid even in the absence of an alkali trap. In the work described below, the disturbing effect of trapping was eliminated by using the free acid throughout. It was shown that the free acids exercised no toxic effect, for the respiration rate was practically unaffected, and the acids were readily assimilated.

EXPERIMENTAL METHODS

All the cultures were prepared from a single strain of *A. niger*, catalogued as S.I. 133 C.

The modified Czapek-Dox synthetic medium used for the growth of the experimental cultures had the composition: 1000 c.c. water; 50 g. glucose, 3·0 g. $NaNO_3$, 1·0 g. $MgSO_4.7H_2O$, 2·0 g. KH_2PO_4, and 0·02 g. $FeSO_4.7H_2O$.

In nearly every case the fungus was reared on 200 or 250 c.c. of the culture medium in 500 c.c. bolt-head flasks. The temperature was maintained at 22° C. A continuous current of CO_2-free air was led over the cultures. The CO_2 output was determined by absorption in Pettenkofer tubes containing caustic soda solution. All parts of the apparatus were sterilized before or during its assembling, and bacteria and spores were removed from the entering air stream by passing it through potash bubblers followed by cotton-wool filters.

In the earlier stages of the investigation, the solutions were unstirred. Later work indicated the desirability of a stirring device, and in the main series of experiments the solutions were continuously stirred. In this group, the flasks were placed with the neck uppermost. The air inlet tube passed almost to the bottom of the flask before turning sharply upwards. Issuing air bubbles thoroughly stirred the surrounding liquid, but there was no disturbance of the mat of mycelium, since the bubbles passed into a glass bell perforated by small holes above the level of the liquid. The air exit tube passed inwards almost to the level of the mycelium, and another tube continuing to the bottom of the flask provided for the supply and removal of the solution investigated. Samples of the solution were removed by 1 c.c. pipettes which were constructed from narrow glass tubing and capped with rubber teats. A rubber collar ensured that the pipette fitted tightly into a wider glass tube passing through the bung.

The experimental procedure closely resembled that of Bennet-Clark & La Touche (1935). When a good growth of mycelium had been obtained, the culture solution was run off, and, after thorough washing of the mat, was replaced by sterile distilled water. The conditions of starvation were maintained for several days before supplying solutions of sugars or organic acids. Samples of the solution were removed for analysis at frequent intervals.

ANALYTICAL METHODS

The titratable acidity of the solution was obtained by titration of the solution with 0·02*N* carbonate-free sodium hydroxide solution.

Any oxalate in these samples was determined by Bau's method. Glucose and other sugars were determined by the micro-carbonate-tartrate reagent of Shaffer and Hartmann.

In the experiments with oxalacetic acid and sodium oxalacetate, pyruvic acid was estimated by the method developed by Raistrick & Birkinshaw (1931, p. 150) for pyruvic alcohol. The 1 c.c. sample removed from the culture vessel was diluted to a measured volume when necessary, and an aliquot part was added to 40 c.c. of $0{\cdot}1N$ iodine + 50 c.c. of $0{\cdot}1N$ sodium hydroxide. The mixture was left for 2 hours, and after addition of 60 c.c. of $0{\cdot}1N$ sulphuric acid, the excess of iodine was titrated with $0{\cdot}1N$ thiosulphate (1 molecule of pyruvic acid $\equiv 6$ I). Oxalacetic acid undergoes the same reaction and was allowed for, when present. In some cases, allowance had to be made for the presence of glucose, which also reacts quantitively with iodine under the conditions described.

Oxalacetic acid was occasionally determined by the iodine method described above, but more frequently a simpler method was adopted. When oxalacetic acid is boiled, one of the carboxyl groups is removed as CO_2 and pyruvic acid is left. The amount of oxalacetic acid present is therefore double the loss of acidity on boiling. It was found that 15 min. boiling was sufficient to drive out the carbon dioxide from the solution. When free base was present, excess of standard sulphuric acid was added. The quantity of oxalacetic acid was determined by back titration with soda on boiled and unboiled samples.

Experimental results

Group 1

The experiments of this group are a repetition of those of Raistrick & Clark (1919). The work was repeated to ensure that the strain of *A. niger* used throughout the present work would show the same remarkable behaviour when reared under the same conditions. In addition, several cultures had the sodium salt replaced by the free acid.

The results were in general agreement with those of the previous workers, but the actual quantity of oxalic acid produced was considerably less. A fair yield of oxalic acid was obtained with sodium citrate, malate and acetate, but greater quantities appeared when calcium gluconate or sodium citrate was used. Oxalic acid was not

obtained from the salts of fumaric, maleic, lactic, pyruvic, and glycollic acids.

When the free acid was used, growth was poor or absent, except in the cases of succinic, fumaric, and citric acids. In the cases of succinic and citric acids, approximately the same amount of oxalic acid was obtained from the free acid and the sodium salt.

Group 2

In the experiments of this group, cultures prepared on the standard medium were starved on distilled water. After several days of starvation solutions of various organic acids were supplied.

(*a*) *Oxalacetic acid.*

Cultures A_1 *and* A_2. These cultures were started at the same time on 100 c.c. of the usual medium. When the rate of CO_2 production passed its maximum value, the culture solution was run off, the mats were washed with distilled water, and each culture was supplied with 100 c.c. of sterile distilled water.

After 4 days on distilled water, A_2 received 100 c.c. of a 0·25 % solution of oxalacetic acid, while A_1 was left on water as a control. A_2 showed a marked increase in the rate of CO_2 production, while the CO_2 output of A_1 gradually decreased. Oxalic acid was not detected in A_2.

Almost all the oxalacetic acid had disappeared at the end of 2 days. The residual solution was run out, the mat was washed with distilled water, and 100 c.c. of 2 % oxalacetic acid was supplied. A marked increase in the CO_2 output resulted, but oxalic acid did not appear.

The results are summarized in Table I and in Fig. 1.

TABLE I. *Oxalacetic and titratable acid losses and gain in* CO_2

	First exposure 0·25 % oxalacetic acid		Second exposure 2·0 % oxalacetic acid	
	11.0 a.m. 15th day to 10.30 a.m. 16th day	10.30 a.m. 16th day to 10.30 a.m. 17th day	1.30 p.m. 17th day to 11.30 a.m. 18th day	11.30 a.m. 18th day to 10.30 a.m. 19th day
Oxalacetic acid loss in mg.-equivalents	1·05	0·65	10·83	1·42
Titratable acid loss in mg.-equivalents	0·67	0·38	8·20	0·60
Additional CO_2 evolved in mg. carbon	9·8	6·4	50·5	13·6

It would appear from these results that the bulk of the oxalacetic acid was decomposed into pyruvic acid and carbon dioxide. If all the oxalacetic acid lost were decomposed in this way, the ratio of the loss of oxalacetic acid to the loss of titratable acid should be 2 : 1. In Table I, the ratio is 2 : 1·27 over the first 23½ hours and 2 : 1·17 over the next 24 hours. These values suggest that part of the oxalacetic acid, or part of the pyruvic acid produced, was utilized by the fungus. This point was brought out more clearly by the second exposure to oxalacetic acid. For the first 22 hours the ratio was 2 : 1·51, while

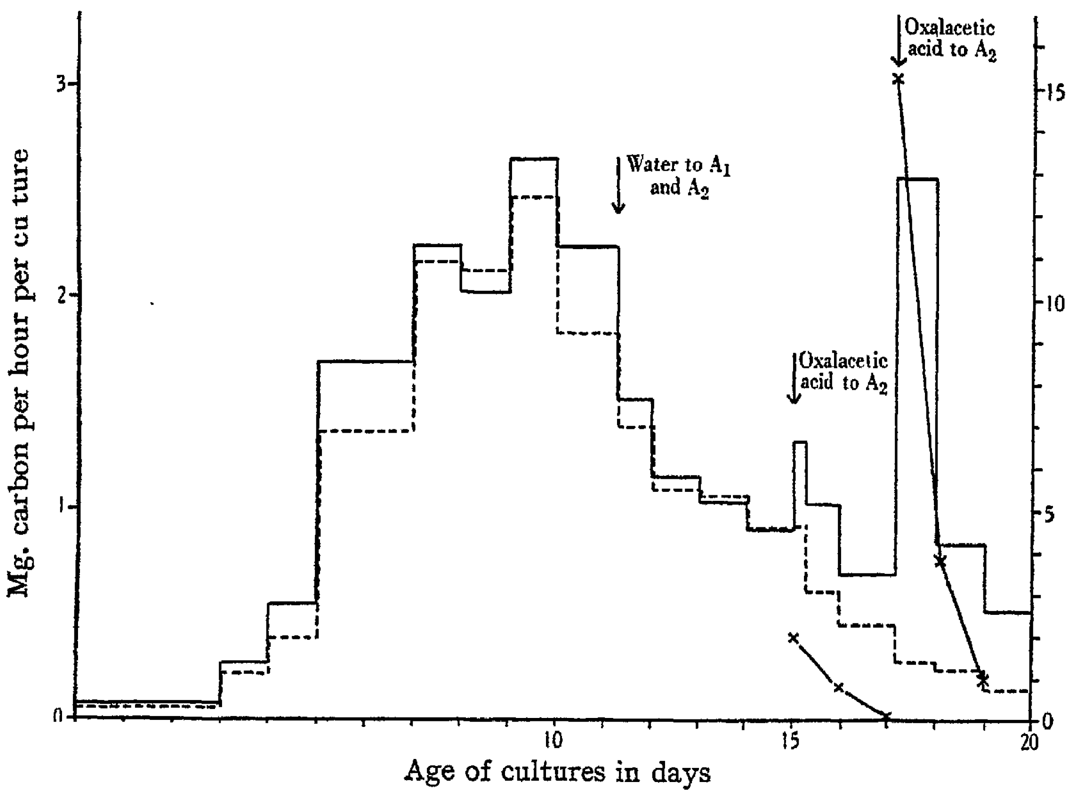


Fig. 1. Respiration-time curves of cultures A_1 (---) and A_2 (—) and acid loss-time curves of A_3. Actual concentrations of oxalacetic acid are given by the record —×—, the ordinates for which are on the right.

over the next 23 hours the ratio was 2 : 0·845. The former value indicates that a considerable portion of either oxalacetic acid or pyruvic acid was used up by the fungus, while the ratio for the last 23 hours was possibly the result of the formation of a small quantity of acid by the fungus.

The carbon dioxide output is in agreement with the view that the oxalacetic acid gives rise to pyruvic acid and carbon dioxide, but indicates that not all the oxalacetic acid can undergo this change. It is shown in Table I that, from 1.30 p.m. on the seventeenth day to 11.30 a.m. on the eighteenth, 10·83 mg.-equivalents of oxalacetic

acid disappear. This amount of oxalacetic acid would yield 65 mg. of carbon as carbon dioxide, while the total carbon dioxide output from the fungus was 56·5 mg. of carbon. An approximately correct figure of extra carbon dioxide output of A_2 may be obtained by subtracting the carbon dioxide output of A_1 from that of A_2. In this way we obtain a maximum additional carbon dioxide output of 50·5 mg. C. This figure is only 77·7 % of that calculated on the assumption that all oxalacetic acid disappearing was converted into pyruvic acid and carbon dioxide. Therefore, at least 22·3 % of the oxalacetic acid must have undergone a change other than "acid hydrolysis" or decomposition into pyruvic acid and carbon dioxide.

Cultures E_1 and E_2. Cultures E_1 and E_2 were grown on 50 c.c. of the culture solution in 100 c.c. bolt-head flasks. On the sixth day the cultures were starved in the usual way. After 4 days of starvation, E_1 was given 50 c.c. of a mixture of sodium oxalacetate and oxalacetic acid, prepared by titrating 0·45 g. of oxalacetic acid with caustic soda to *p*H 7. Sodium oxalacetate alone would have been harmful on account of its alkalinity. E_2 was left on water.

No marked rise was noted in the CO_2 output of E_1, and none was to be expected, since any carbon dioxide arising by the decomposition of the oxalacetic acid would be fixed as sodium carbonate. Oxalic acid was not detected till 3 days had elapsed since the supply of the oxalacetate mixture, and by this time nearly all the oxalacetic acid had disappeared. A sample of the oxalacetate mixture, which had been placed in a test-tube as a control, showed a slower rate of decomposition than the solution supplied to the fungus. The actual figures are given in Table II.

TABLE II

	Age of culture and time of determinations				
	10th day, 3.0 p.m.	11th day, 11.0 a.m.	12th day, 11.0 a.m.	12th day, 5.0 p.m.	13th day, 11.0 a.m.
Oxalacetic acid loss in mg.-equivalents	—	2·64	1·49	0·40	0·44
Oxalacetic acid concentration in mg.-equivalents per 100 c.c.	14·0	8·45	4·97	3·90	2·33
Oxalacetic acid concentration of *control* in mg.-equivalents per 100 c.c.	14·0	9·26	6·48	—	5·58

The oxalic acid appearing on the third day after the supply of the oxalacetate almost certainly originated from the carbohydrate reserves of the mycelium, for no oxalic acid was obtained during the first 2 days, when the rate of oxalacetate disappearance was at its highest. A *p*H determination on the second day revealed that the alkalinity of the medium had increased to *p*H 8·4. It is probable that the oxalic acid was produced as a result of the presence of free base generated by the utilization of the organic anions.

TABLE III. *Changes in concentration of oxalacetic acid, titratable base, pyruvic acid, and oxalic acid in culture E_3, and a test-tube control*

	Age of culture and time of determination				
Control	6th day, 11.40 a.m.	7th day, 10.30 a.m.	8th day, 11.0 a.m.	9th day, 11.0 a.m.	10th day, 11.0 a.m.
Oxalacetic acid in mg.-equivs. per 100 c.c.	9·43	6·90	5·66	5·25	3·04
Titratable base in mg.-equivs. per 100 c.c.	0·49	1·31	1·92	2·13	3·24
Culture E_3					
Oxalacetic acid in mg.-equivs. per 100 c.c.	9·50	6·40	4·00	3·93	0·99
Titratable base in mg.-equivs. per 100 c.c.	0·18	1·78	2·30	1·03	0·29
Pyruvic acid in mg.-equivs. per 100 c.c.	0·01	1·70	3·00	1·08	1·02
Oxalic acid in mg.-equivs. per 100 c.c.	0	0	0	2·06	5·60
Oxalacetic acid loss in mg. carbon	—	34·6	23·1	0·65	20·0
Pyruvic acid gain in mg. carbon	—	26·3	18·7	−23·4	−0·66
Oxalic acid gain in mg. carbon	—	0	0	8·38	12·10

Culture E_3. This culture was grown under the same conditions as E_1 and E_2. The fungus was starved on the fifth day, and on the following day the water was replaced by 50 c.c. of a 0·63 % solution of "sodium oxalacetate". As in the case of E_2, a sample of the oxalacetate was set aside as a control. The results are given in Table III and Fig. 2.

For the first 2 days the loss of oxalacetic acid carbon and the gain in pyruvic acid carbon coincided closely with the 4 : 3 ratio demanded by the "ketonic hydrolysis" of the oxalacetate. Oxalic

acid appeared on the third day, but from 11.0 a.m. on the eighth day of growth to 11.0 a.m. on the ninth day, the oxalic acid carbon gain was nearly thirteen times greater than the oxalacetic acid carbon loss. There is little doubt that this oxalic acid was produced from substances stored within the mycelium.

The above experiments show that the bulk of the oxalacetic acid supplied to the fungus is decomposed into pyruvic acid and

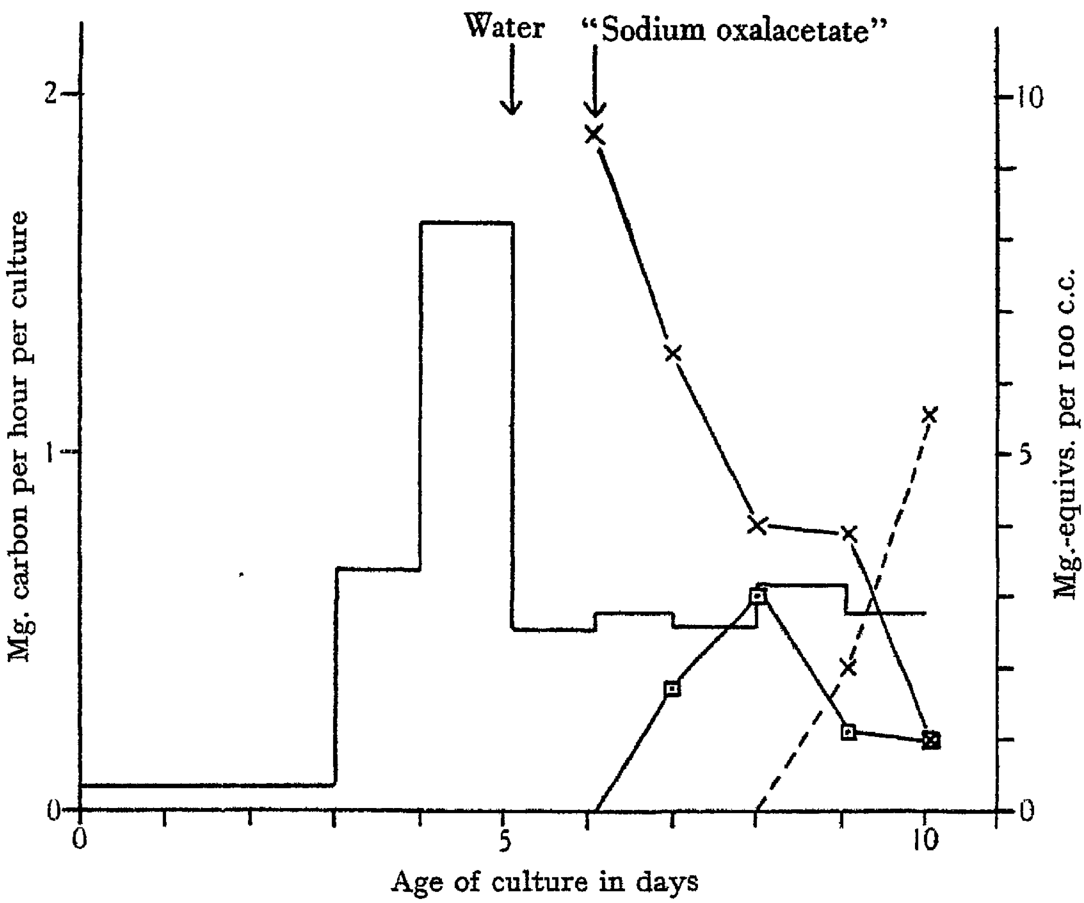


Fig. 2. Progress curves for culture E_3. The CO_2 output in mg. carbon per hour is given by the stepped graph. The concentrations of oxalic acid (curve --×--), pyruvic acid (curve —⊡—) and oxalacetic acid (curve —×—) are given in mg.-equivs. per 100 c.c. of culture fluid, with ordinates on the right.

carbon dioxide. Oxalic acid is not produced when the free acid is supplied, while the oxalic acid produced in the presence of sodium ions is almost certainly derived from the contents of the mycelium. Wieland (1924) showed that the loss of carbon dioxide from solutions of oxalacetic acid is accelerated by surface catalysts and dead organic matter. In the experiments described above, it is possible that a large proportion of the oxalacetic acid was decomposed on the surface of the mycelium without actually entering the hyphae, but in the case

of A_2, at least 22·3 % of the oxalacetic acid had entered the fungus, for that loss could not be accounted for by the "ketonic hydrolysis".

(*b*) *Malic acid.*

Culture A_2, previously used in section (*a*), was washed several times with distilled water and supplied with 100 c.c. of a 1 % solution of malic acid. The experiment was continued for 16 days and aliquot parts of the solution were removed for analysis every 2 or 3 days. Over the major part of this period, the malic acid loss in mg. carbon per hour exceeded the CO_2 output in mg. carbon per hour, but oxalic acid was not produced.

The results are recorded graphically in Fig. 3.

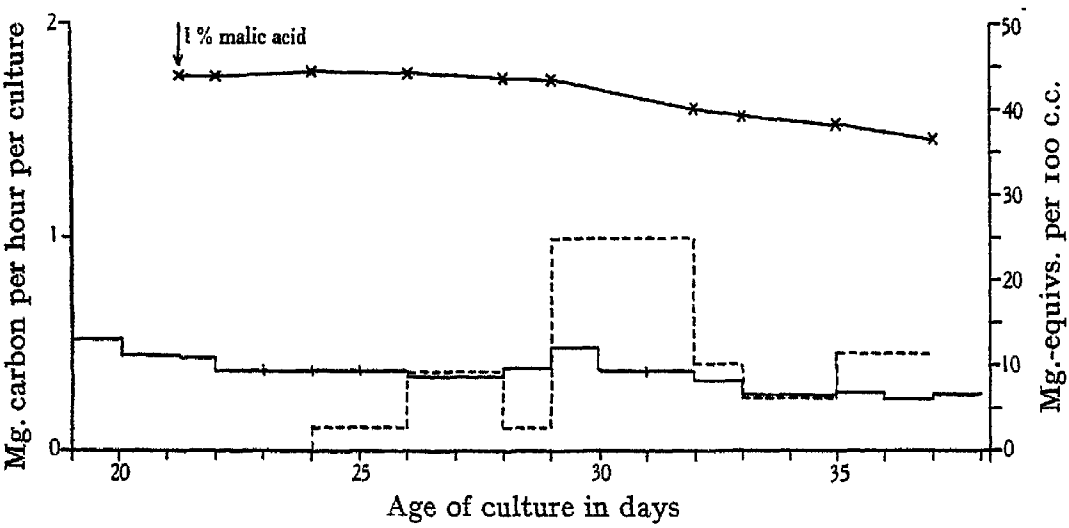


Fig. 3. Respiration-time and acid loss-time curves for culture A_2. The CO_2 output (——) and the acid loss (– – – –) are given in mg. carbon per hour by the stepped graphs. The actual concentrations of malic acid are given by the record —×— with ordinates on the right.

(*c*) *Succinic acid.*

Culture B_2 was grown on 200 c.c. of culture solution for 10 days. The culture was then starved for 6 days and 200 c.c. of an approximately 1 % succinic acid solution were supplied.

The results are given in Fig. 4.

It will be noticed that for the first 14 days the acid carbon loss exceeded the CO_2 carbon loss. Over this period oxalic acid was not detected, but later on small quantities of oxalic acid appeared.

(*d*) *Pyruvic acid.*

Culture B_1 was grown under the same conditions as B_2. After a period of starvation, a 2·6 % solution of pyruvic acid was supplied. An increase in the CO_2 output suggested that some of the acid was

decarboxylated, but all the acid could not have undergone this change, for over a period of 24 hours the pyruvic acid carbon loss was over four times greater than the total CO_2 output. Readings were continued for 4 days, but no oxalic acid appeared.

(*e*) *Gluconic acid.*

Culture F_1. This culture was reared on 50 c.c. of culture solution in a 100 c.c. bolt-head flask. On the fifth day the culture solution was run off and replaced by distilled water, and on the following day 50 c.c. of a 1·25 % solution of gluconic acid were supplied.

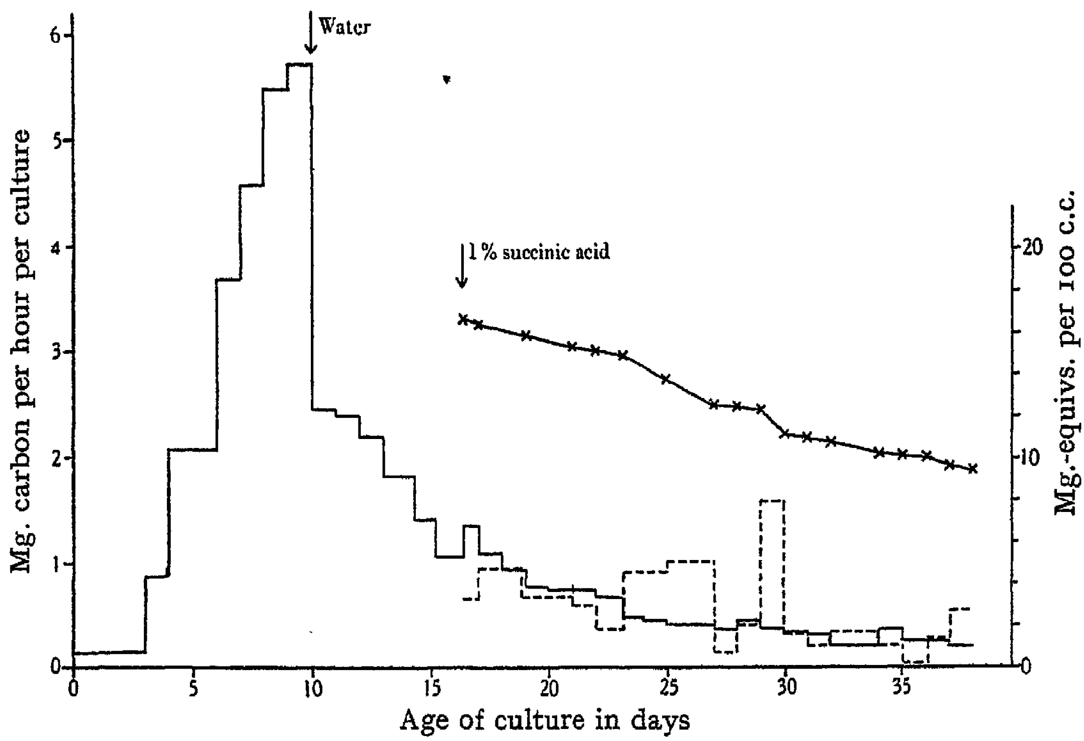


Fig. 4. Respiration-time and acid loss-time curves for culture B_2. Concentrations of succinic acid are given by the curve —×—, with ordinates on the right.

Over the period of the experiment, 4 days, there was a slow increase in titratable acidity. Oxalic acid was first detected as a precipitate on the third day. On the fourth day the following values were obtained:

Oxalic acid in mg.-equivs. per 100 c.c. ...	1·61
Titratable acid in mg.-equivs. per 100 c.c. ...	7·20
Gluconic acid in mg.-equivs. per 100 c.c. ...	5·59
Gluconic acid loss in mg.-equivs. per 100 c.c. ...	0·79

Thus 0·79 mg.-equivs. of gluconic acid were lost and 1·61 mg.-equivs. of oxalic acid were formed. This ratio 1 : 2·04 mg.-equivs.

appeared to have possible significance. Further experiments were carried out to test this point.

Cultures M_1, M_2, M_3 and M_4. These cultures were prepared on 200 c.c. of the standard medium in 500 c.c. bolt-head flasks. After 6 days of growth, the cultures were starved on distilled water for 5 days. Each culture then received 200 c.c. of approximately 1 % gluconic acid.

The results obtained with M_1 and M_2 are given in Figs. 5 and 6. These cultures were chosen for graphical illustration as representing two extremes, and the graphs of M_3 and M_4 differ from them only in detail. It will be seen that the addition of gluconic acid was followed by a slight increase in the CO_2 output. This rise occurred in every case. Except in M_1, there was no appreciable loss of gluconic acid (curve —⊡—) for about 6 days, but considerable quantities of oxalic acid (curve —×—) appeared. It should be noted, however, that the amount of oxalic acid was not in excess of the quantity produced on distilled water in other experiments (see Figs. 9 and 11). M_1 was exceptional in showing a disappearance of gluconic acid after 3 days (see Fig. 5). During the last 4 days of the experiment gluconic acid disappeared rapidly in every case and oxalic acid accumulated. In Figs. 5 and 6 stepped graphs represent the gluconic acid loss and oxalic acid gain in mg. of carbon per hour, oxalic acid being represented by broken lines, and gluconic acid by the ·—·—·— record.

TABLE IV. *Gluconic acid losses and gains in oxalic acid*

	M_1	M_2	M_3	M_4
Mg.-equivs. of total oxalic acid gain per mg.-equivs. of total gluconic acid loss	1·37	2·15	1·01	1·69
Molecules of oxalic acid produced per molecule of gluconic acid:				
(*a*) From 10.15 a.m. on 17th day of growth to 10.15 a.m. of 19th day	0·39	0·42	0·40	0·44
(*b*) From 10.15 a.m. on 19th day of growth to 10.15 a.m. on 21st day	0·36	0·41	0·38	0·54

The ratios of the total gain in oxalic acid to the total loss of gluconic acid, both expressed as milligram equivalents, are given for each culture in Table IV. Table IV also includes the gluconic acid : oxalic acid ratios for the last 4 days of the experiment, the results being expressed here as molecules of oxalic acid produced for the loss of 1 molecule of gluconic acid.

In the Discussion, where the results are considered at greater length, it is suggested that gluconic acid is an intermediate product

in the formation of oxalic acid from glucose, one molecule of gluconic acid giving rise to one of oxalic acid. The values obtained in the above

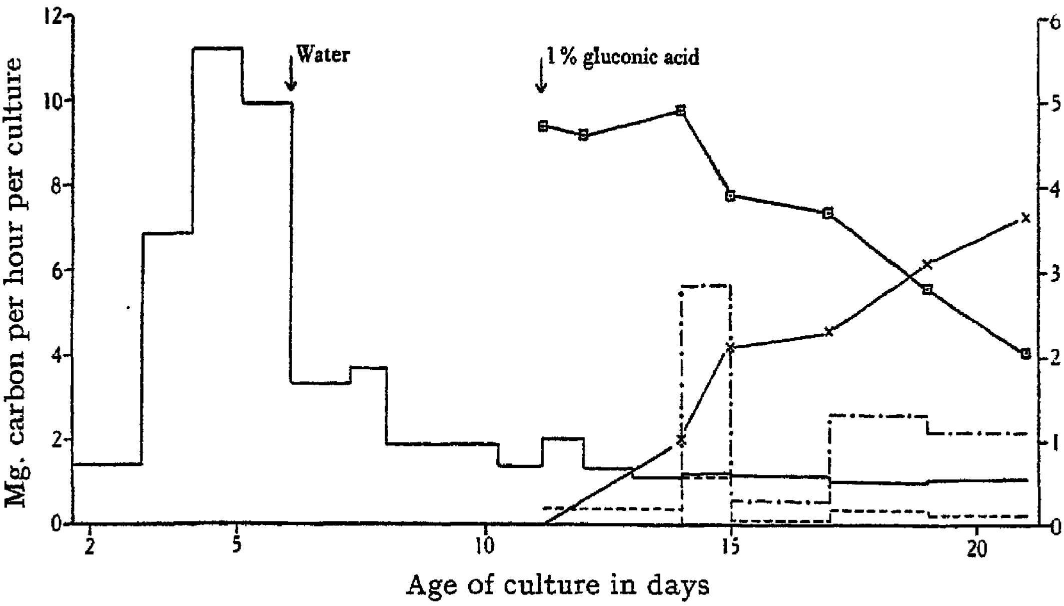


Fig. 5. Progress curves for culture M_1. CO_2 output (——), gluconic acid loss (—·—·) and oxalic acid gain (– – – – – –), all in mg. carbon per hour, are represented by the stepped graphs. Actual concentrations of oxalic acid and gluconic acid are given by the curves —×— and —⊡— respectively, the ordinates for which are on the right.

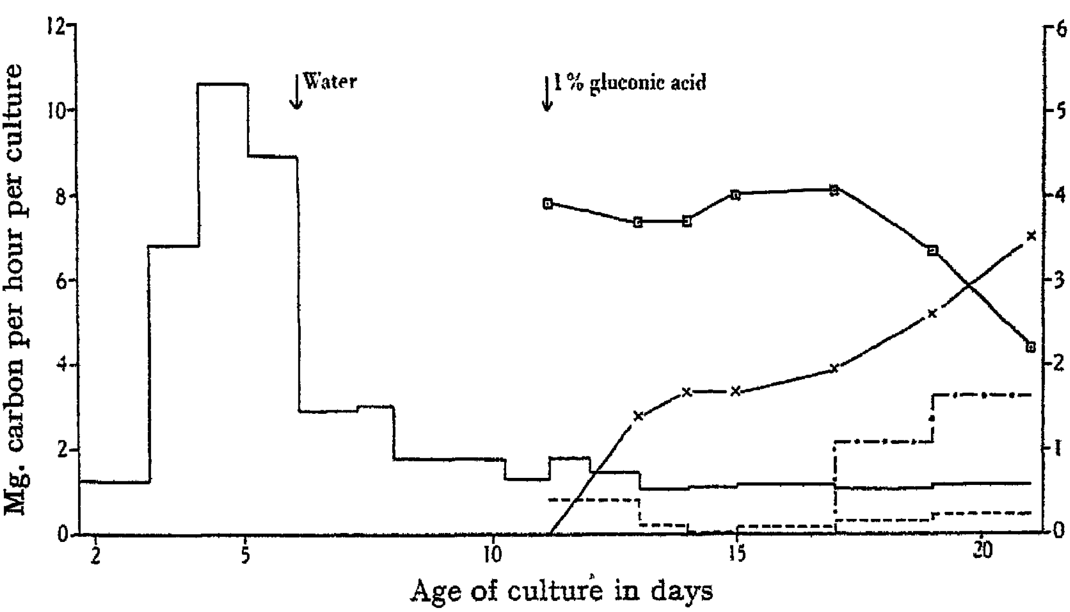


Fig. 6. Progress curves for culture M_2. The records of CO_2 output, gluconic acid loss, oxalic acid gain, and actual concentrations of the acids are represented exactly as in Fig. 5.

experiment, however, indicate that less than half a molecule of oxalic acid is accumulated from 1 gluconic acid molecule. These results could be explained either by the utilization of some of the gluconic

acid in a different manner, or by the removal of a proportion of the oxalic acid. The former explanation is rendered unlikely by the fact that the gluconic acid concentration remains approximately constant for several days. Support for the second explanation is afforded by the increase in the respiration rate which follows the supply of gluconic acid. Even if the disappearing oxalic acid were completely converted into CO_2 (which is by no means certain) the CO_2 output would still be in agreement with the CO_2 values actually obtained in our experiments.

Group 3

The formation of oxalic acid from sugars.

In the experiments described below cultures were supplied with solutions of several pentoses and hexoses.

Cultures L_1, L_3, L_5, L_6 *and* L_7 were reared on 250 c.c. of the culture solution in 500 c.c. bolt-head flasks. After 9 days of growth the cultures were starved for 2 days. At the end of this time the water was run off, the mats were washed, and each culture given 200 c.c. of an approximately 1 % solution of the sugar under investigation. L_1 received arabinose, L_3 xylose, L_5 fructose, L_6 glucose, and L_7 galactose.

In every case, addition of the sugar solution was followed by an increase in the rate of CO_2 production. Oxalic acid appeared on the first day. Parallel determinations of titratable acid and oxalic acid showed that in each instance the acid formed was nearly pure oxalic acid. It was found that glucose was used up more rapidly than the other sugars. Fructose, arabinose, and xylose were also readily utilized, but galactose disappeared at a slower rate. The glucose and xylose results are given in Figs. 7 and 8 respectively. The results with the other sugars are not figured but were essentially similar.

In the present section many experiments were carried out on the production of oxalic acid from glucose. In some cases the fungus was reared throughout the experiment on the original Czapek solution, while in others the mycelium was starved on water and later supplied with pure glucose solutions.

Culture J_1. This culture formed one of a group of five, the conditions of growth of which are described in Group 4, Series *J*, p. 348. The results of this typical experiment are given in Fig. 9. The CO_2 output, glucose loss and oxalic acid gain are expressed as mg. carbon per hour and are shown by the stepped graphs, the CO_2 output being represented by a continuous line, the oxalic acid gain by a broken

line, and the glucose by the dot-dash line. The oxalic acid concentration is given in mg.-equivs. per 100 c.c. by the curve —×—, while the glucose concentration is given in g. per 100 c.c. by the curve —⊡—.

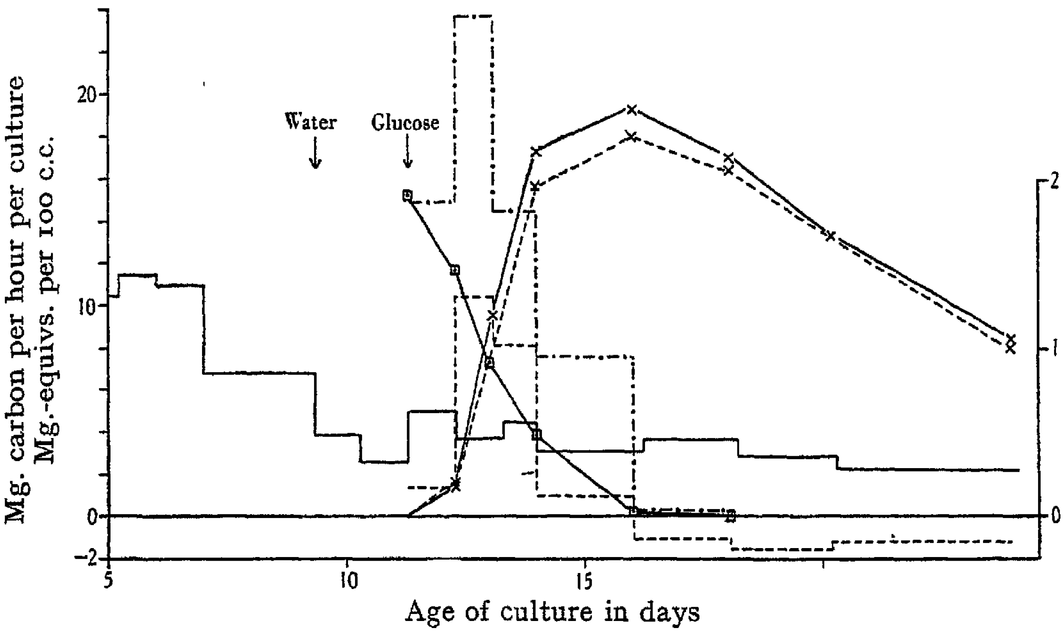


Fig. 7. Respiration-time curves of culture L_6. The stepped graphs record the CO_2 output (——), glucose loss (—·—·—·) and oxalic acid gain (– – – – – –) in mg. carbon per hour. Concentrations of titratable acid and oxalic acid are given by the curves —×— and – – –×– – – respectively, the ordinates for which are on the left. The actual glucose concentration is shown by the record —⊡— with ordinates on the right.

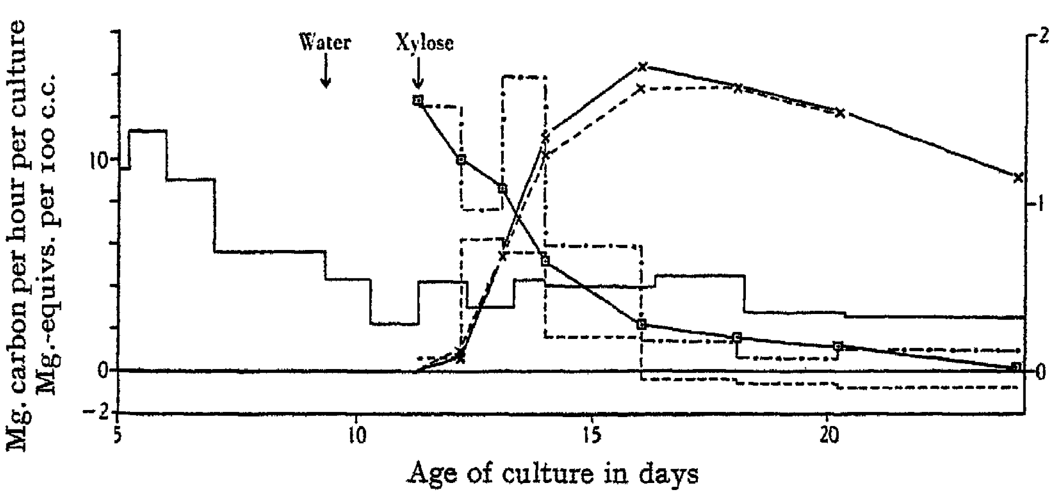


Fig. 8. Respiration-time curves of culture L_3. In this case curve —⊡— represents the xylose concentration while the xylose loss in mg. carbon per hour is shown by the record —·—·. Otherwise the curves are represented exactly as in Fig. 7.

It will be seen that for several days there is no increase in the titratable acidity, but with further growth oxalic acid appears, the rate of acid production rising to a maximum value. In the particular

experiment described, glucose determinations were not carried out over the early period of growth, but it is found that in other cases the maximum rate of glucose loss is usually attained at approximately the same time as the maximum rates of CO_2 production and oxalic acid formation. During this period the glucose carbon loss is invariably far in excess of the quantities of carbon produced as CO_2 and oxalic acid.

In the present case, at the point indicated in the figure by the first arrow, the culture solution was run off, and distilled water was

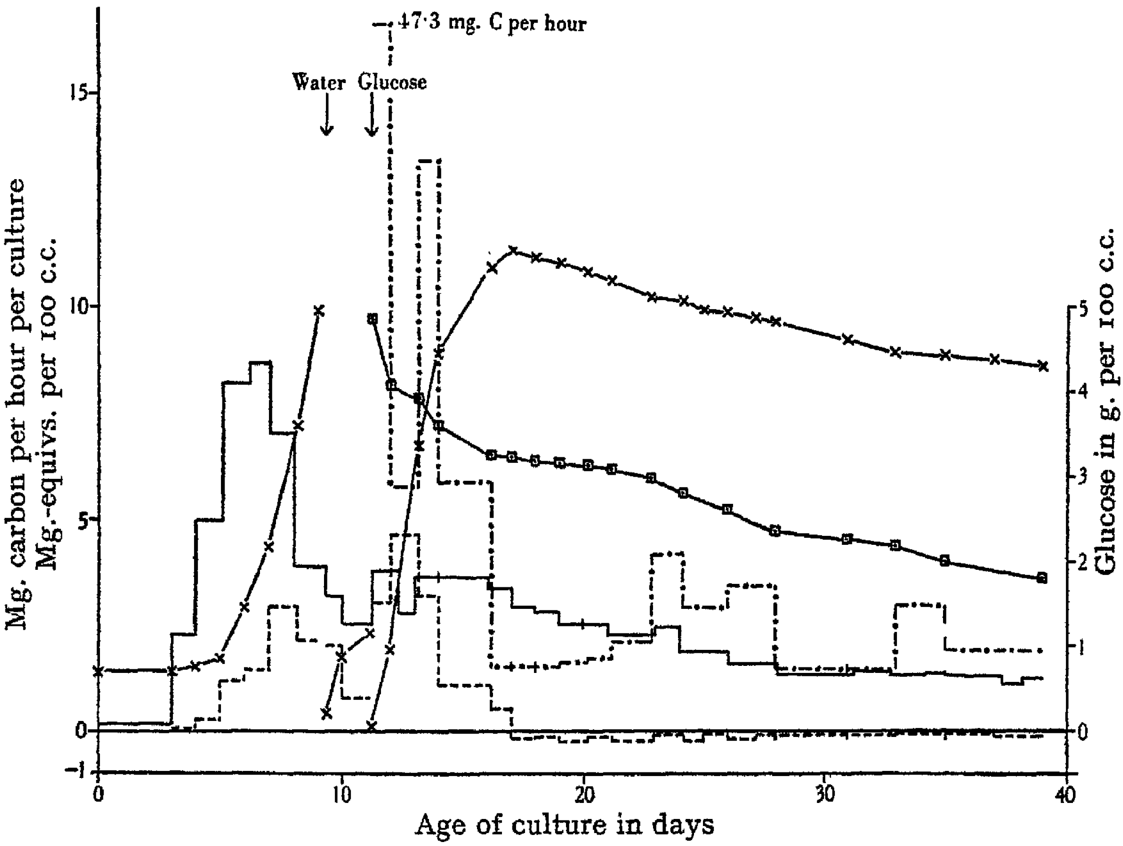


Fig. 9. Respiration-time curves of culture J_1. The representation corresponds exactly with that of Fig. 7.

supplied. It is shown in the figure that oxalic acid continues to accumulate, evidently arising from the carbohydrate reserves of the mycelium. Similar results are shown in Figs. 10 and 11. It was thought at first that the oxalic acid produced during starvation might owe its origin to traces of sugar that washing had failed to remove, but other experiments revealed that replacement of the acid water by a fresh supply of distilled water resulted in the production of an additional amount of oxalic acid (see Figs. 10 and 11). It is particularly striking in view of these results, that not even a trace

of oxalic acid is detected when the starved fungus is supplied with solutions of organic acids.

After 2 days of starvation, culture J_1 was supplied with 4·8 % glucose solution (second arrow in Fig. 9). The addition of glucose produced a small but definite increase in the CO_2 output. The supply of sugars was invariably followed by such a rise in the respiration

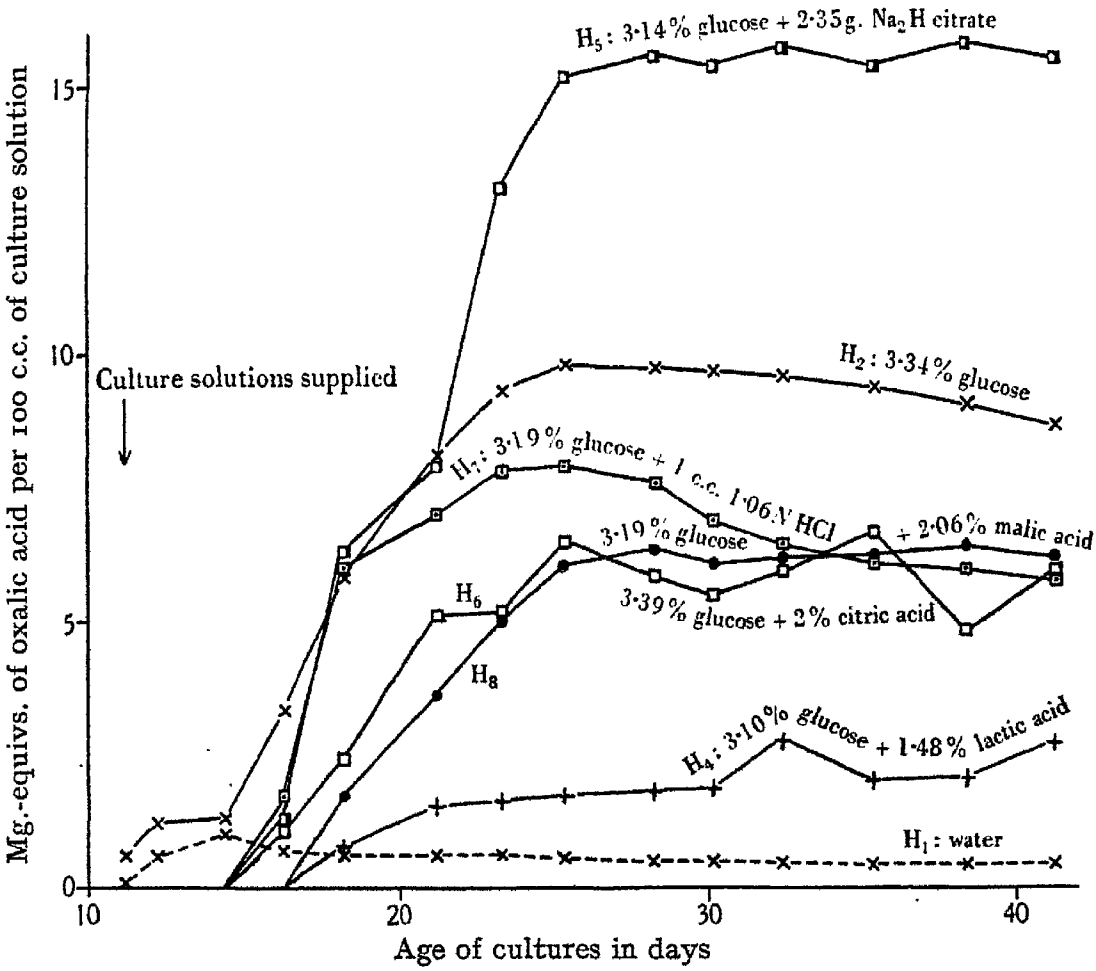


Fig. 10. Progress curves of the production of oxalic acid by cultures supplied with: H_1, water (curve ---×---); H_2, 3·34 % glucose (curve —×—); H_4, 3·10 % glucose+1·48 % lactic acid (curve —+—); H_5, 3·14 % glucose+2·35 g. disodium hydrogen citrate (curve —◨—); H_6, 3·39 % glucose+2 % citric acid (curve —□—); H_7, 3·19 % glucose+1 c.c. 1·06N HCl (curve —⊡—); H_8, 3·19 % glucose+2·06 % malic acid (curve —●—).

rate. It is shown in the figure that oxalic acid rapidly accumulates to a high concentration, but after several days the acid commences to disappear at a slow but steady rate, although a large quantity of glucose still remains unused by the fungus.

The disappearance of oxalic acid in the presence of sugar was found in every case in which high concentrations were used, but this effect

was not apparent when the sugar concentration was low (as in Figs. 7 and 8).

At the beginning of the investigation it was anticipated that the carbon ratio of the glucose loss, oxalic acid gain, and CO_2 output, might afford some insight into the nature of the breakdown of the glucose molecule. In none of the experiments, however, was a constant numerical relationship detected. This is clearly shown by Figs. 7,

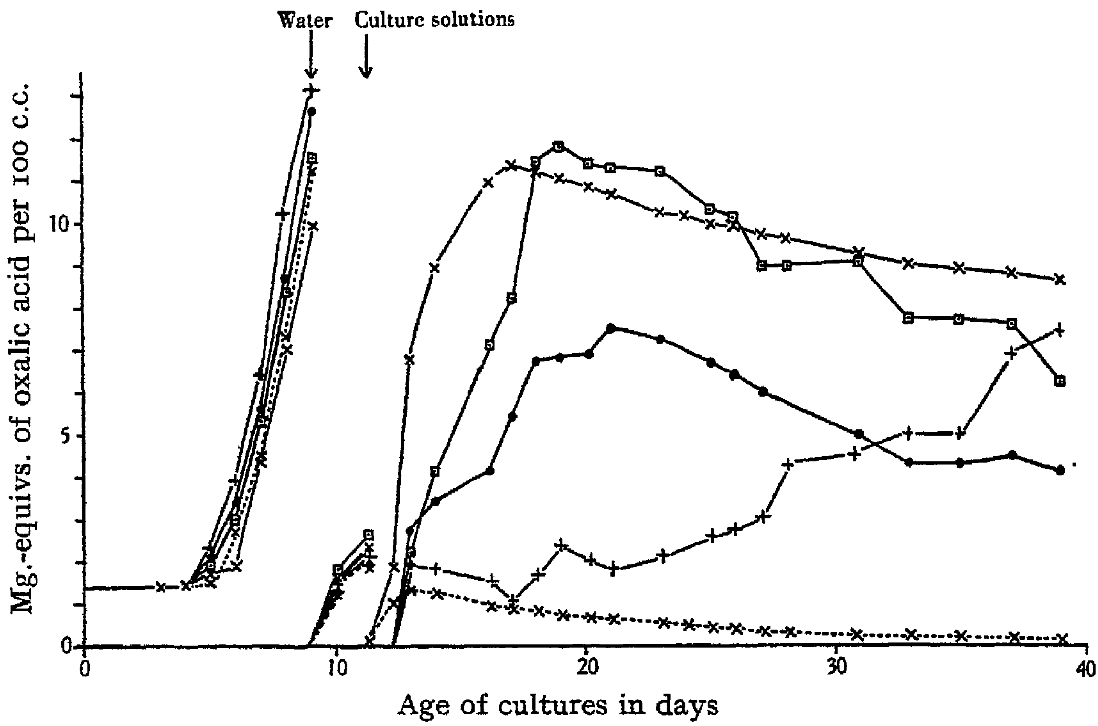


Fig. 11. The quantities of oxalic acid in 100 c.c. of culture solution in J_1 (curve —×—), J_2 (curve —●—), J_3 (curve —+—), J_4 (curve - - - × - - -) and J_5 (curve —⊡—). Till the date indicated by the first arrow the curves represent the production of oxalic acid on the standard culture solution. The curves for the period between the first and second arrows give the quantities of oxalic acid formed during starvation. At the second arrow the cultures were supplied with: J_1, 4·83 % glucose; J_2, 4·65 % glucose +0·94 % pyruvic acid; J_3, 4·4 % glucose+1·05 % lactic acid; J_4, distilled water; J_5, 4·5 % glucose+0·78 % glycollic acid, and the curves record the quantities of oxalic acid produced in these solutions.

8 and 9, in which the oxalic acid carbon gain is in excess of the CO_2 output for a short period, but rapidly diminishes without any sudden fall in the respiration rate.

Culture G_1. In this experiment the fungus was supplied repeatedly with solutions of pure glucose, a period of starvation preceding each addition of glucose solution. The results revealed a slowing down of oxalic acid formation with increasing age, but the quantity of oxalic acid formed from a given amount of glucose remained approxi-

mately the same, for although the acid was produced at a slower rate, a correspondingly long period elapsed before it started to disappear.

The experiments outlined in this section emphasize that oxalic acid is not merely the end product of a simple series of chemical reactions, but is the result of a process closely dependent on the vital activity of the fungus.

Group 4

The effect of organic acids on the formation of oxalic acid from glucose.

Results obtained in the present group of experiments show that several organic acids inhibit the production of oxalic acid from glucose.

In the experiments described in Group 5, it was particularly striking that, although growth was exceptionally heavy when the fungus was reared on lactates, not the slightest trace of oxalic acid could be detected. This observation suggested the supply of a mixture of glucose and lactic acid to a starving culture.

Culture C_1. The culture was grown on 200 c.c. of culture solution in a 500 c.c. flask. The fungus was starved on the ninth day of growth, and on the twenty-second day received 200 c.c. of a 2·5 % glucose solution containing 1·89 g. of lactic acid.

Both lactic acid and glucose disappeared at a fairly high rate, the lactic acid carbon loss at some periods exceeding the CO_2 output, but oxalic acid was not detected at any time in the course of the experiment.

Series H. Cultures H_1, H_2, H_4, H_5, H_6, H_7 and H_8 were reared on 200 c.c. of the standard medium in 500 c.c. flasks. After 8 days the fungi were starved. On the third day of starvation the seven cultures were supplied with 200 c.c. of: H_1, water; H_2, 3·34 % glucose; H_4, 3·10 % glucose + 1·48 % lactic acid; H_5, 3·14 % glucose + 2·35 g. disodium hydrogen citrate; H_6, 3·39 % glucose + 2 % citric acid; H_7, 3·19 % glucose + 1 c.c. 1·06*N* HCl, *p*H 2·8; and H_8, 3·19 % glucose + 2·06 % malic acid.

Glucose, titratable acid, oxalic acid and CO_2 were all determined, but only the oxalic acid values are given in Fig. 10.

The figure shows that when malic (curve —●—) and citric acids (curve —□—) were present, the quantity of oxalic acid produced was only slightly less than that from pure glucose (curve —×—). More oxalic acid was produced from glucose + disodium hydrogen citrate (uppermost curve) than from glucose alone, the additional oxalic acid probably owing its origin to the trapping action of sodium

ions. In the case of glucose+lactic acid (curve —+—) the amount of oxalic acid was only a fifth of that produced from glucose.

It was evident that the lactic acid effect was not merely a result of the low *p*H, since the culture supplied with glucose+hydrochloric acid (curve —⊡—) produced almost as much oxalic acid as that reared on pure glucose. In every case the glucose carbon loss was at least equal to the combined losses of carbon as CO_2 and oxalic acid. The production of oxalic acid on distilled water is shown by the lowermost curve in the figure.

Series J. Cultures J_1, J_2, J_3, J_4 *and* J_5 were grown under the same conditions as Series *H*. On the ninth day of growth the culture solutions were replaced by distilled water, and after 2 days of starvation each culture received 230 c.c. of the solution under investigation. J_1 was given 4·83 % glucose; J_2, 4·65 % glucose +0·94 % pyruvic acid; J_3, 4·4 % glucose+1·05 % lactic acid; J_4, distilled water; and J_5, 4·5 % glucose+0·78 % glycollic acid.

As in the case of Series *H*, only the oxalic acid results are given in Fig. 11.

It will be seen from Fig. 11 that, over the period before starvation, there is a close correspondence between the amounts of oxalic acid produced by the different cultures. This parallelism is even more clearly shown by the quantities of oxalic acid formed during the starvation phase.

The supply of solutions of glucose+organic acids, however, gave remarkably different results. In the case of lactic acid (curve —+—) the proportion of oxalic acid was again about one-fifth of the yield from glucose (curve —×—). Glycollic acid (curve —⊡—) was utilized so rapidly that the inhibiting effect was hardly apparent, although the formation of oxalic acid was delayed for a day, while with pyruvic acid (curve —●—) only two-thirds of the quantity of oxalic acid produced from glucose was obtained.

Series K. The results obtained in this section revealed that lower oxalic acid yields in the presence of lactic acid were not to be ascribed to an increase in the rate of disappearance of oxalic acid.

Cultures K_2, K_3, K_4 *and* K_5 were grown on 250 c.c. of the usual culture solution. On the tenth day the cultures were starved on distilled water.

After 3 days of starvation each culture received 250 c.c. of the following solutions: K_2, 4·40 % glucose+1·33 % lactic acid+ 1·27 % oxalic acid; K_3, 4·31 % glucose+1·36 % oxalic acid; K_4, 4·42 % glucose; and K_5, 1·29 % oxalic acid+1·21 % lactic acid.

The results are given in Fig. 12. It is seen that the oxalic acid loss in K_2 (curve —×—) is no greater than that of K_3 (curve —●—), although the former was supplied with glucose + oxalic acid + lactic acid while the latter received only glucose + oxalic acid. It will be noticed that oxalic acid disappeared rather more slowly from K_5 (curve —⊡—), which received no glucose, than from the cultures on

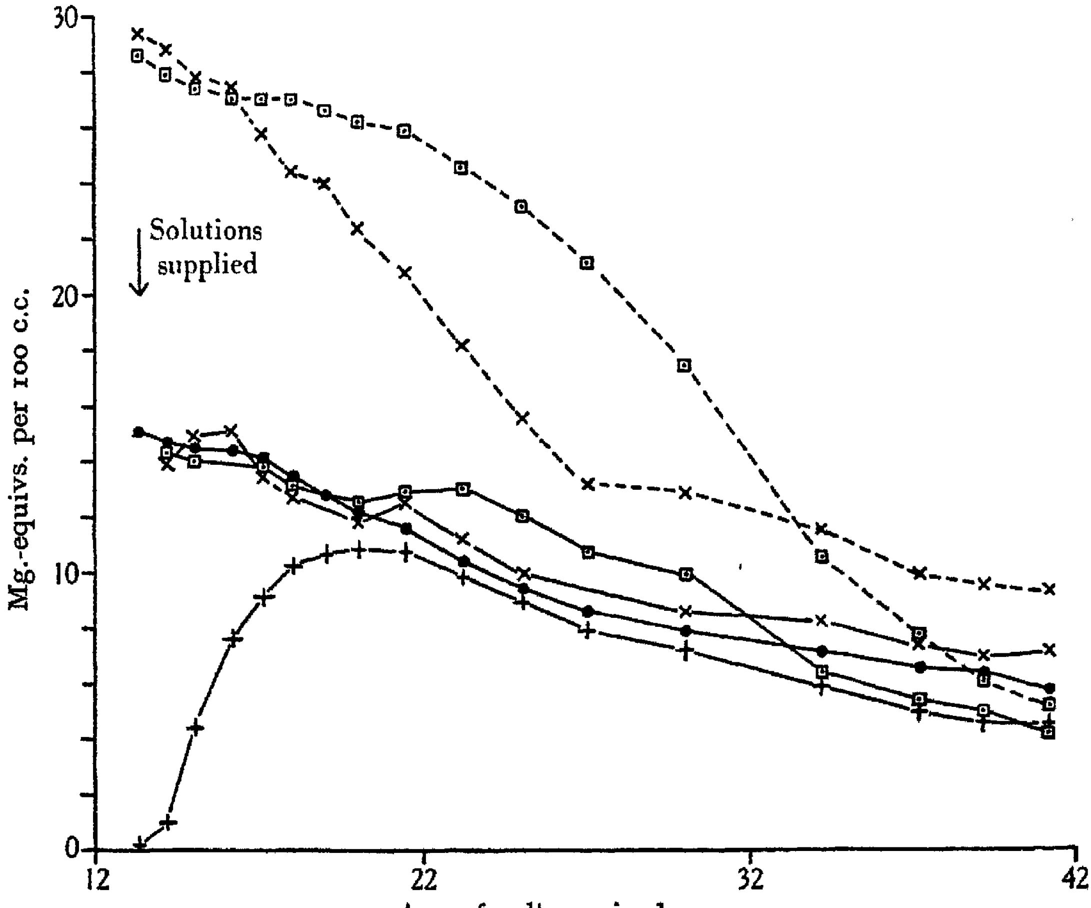


Fig. 12. Progress curves for cultures supplied with: K_2, 4·40 % glucose + 1·33 % lactic acid + 1·27 % oxalic acid; K_3, 4·31 % glucose + 1·36 % oxalic acid; K_4, 4·42 % glucose; and K_5, 1·29 % oxalic acid + 1·21 % lactic acid. The oxalic acid concentrations of K_2, K_3, K_4 and K_5 are given by the curves —×—, —●—, —+—, and —⊡— respectively. The total titratable acidity (i.e. lactic + oxalic acid) is represented for culture K_2 by curve – – –×– – – and for K_5 by curve – – –⊡– – –.

which glucose was also present. The greater rate of acid utilization in the presence of glucose is shown more strikingly when the lactic acid losses are also considered. In the figure curve – – –×– – – represents the loss of titratable acid (i.e. oxalic acid + lactic acid) from K_2, while curve – – –⊡– – – is the loss of oxalic acid + lactic acid from culture K_5. It is clearly shown that the presence of glucose in the former case markedly accelerates the rate of acid disappearance.

An additional point revealed by Fig. 12 is the tendency for the oxalic acid to attain an equilibrium concentration. The figure shows that before there is any disappearance of oxalic acid from culture K_4 the concentration of oxalic acid rises from zero to the level attained by the loss of oxalic acid from the other three cultures.

The significance of these and other results of the present group of experiments is considered again in the Discussion.

Culture K_1. This culture was reared from inoculation on 250 c.c. of a 4·5 % glucose and 1·51 % lactic acid solution. Conidium formation was more prolific than in cultures K_2, K_3, K_4 and K_5.

The formation of oxalic acid was delayed for several days, but as soon as the lactic acid had been reduced to a low concentration, oxalic acid was formed at an increasing rate, till the final yield was as great as that from pure glucose.

DISCUSSION

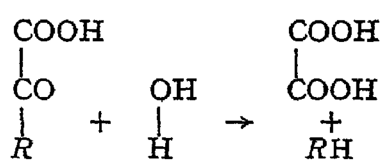
It is widely accepted that the oxalic acid produced by mould fungi arises by the acid hydrolysis of oxalacetic acid. If this view be true, it would be expected that a high yield of oxalic acid would be obtained if the fungus were supplied with a solution of oxalacetic acid. But it has been shown in the experimental part of this paper that the supply of oxalacetic acid to the cultures does not result in the formation of oxalic acid. The amount of oxalic acid produced when the sodium oxalacetate was added bore no direct relation to the quantity of oxalacetate disappearing, and was to be referred to the trapping effect of the free sodium ions. The oxalacetic acid was decomposed mainly into pyruvic acid and carbon dioxide, but a small proportion underwent some other change. These results are in agreement with those obtained from yeast by Neuberg & Gorr (1924), who found that the bulk of the oxalacetic acid yielded carbon dioxide and pyruvic acid, but a certain amount was directly reduced to malic acid.

In our experiments, it was not clearly shown to what extent the decomposition of the oxalacetic acid was due to the vital activity of the fungus. It is possible that the greater part was decomposed on the large surface of the mycelium without actually entering the hyphae. But in one case 22·3 % of the disappearing oxalacetic acid was not removed as pyruvic acid and CO_2; therefore at least that quantity had passed into the hyphae. These results, while not conclusive, render it improbable that oxalacetic acid is an intermediate in oxalic acid formation. This view is supported by an indirect line of evidence. It is generally assumed that the first stage in the utiliza-

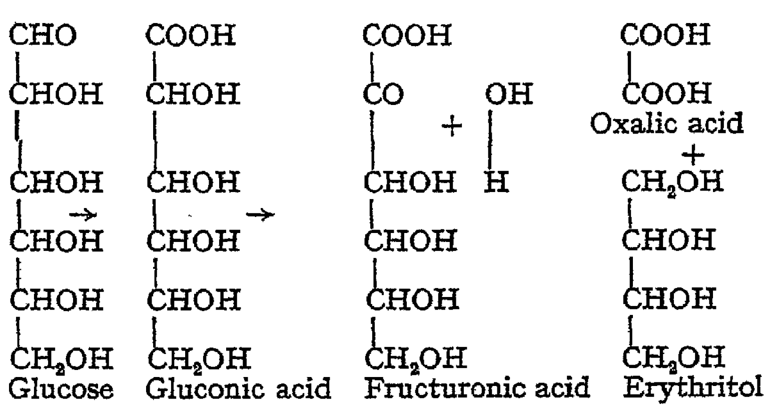
tion of malic acid is its oxidation to oxalacetic acid. If this be true, oxalic acid should be obtained, when malic acid is supplied to the fungus, if oxalacetic acid is the precursor of oxalic acid. Actually, malic acid disappeared fairly rapidly when supplied to culture A_2, but oxalic acid was not produced.

If the hypothesis of the acid hydrolysis of oxalacetic acid be rejected, one is faced with the difficulty of finding an alternative explanation of oxalic acid formation. It has been shown (see Introduction) that oxalic acid is not produced by the direct oxidation of acetic acid. The 3- and 4-carbon acids, and citric acid also, fail to yield oxalic acid. In the present work, when the cultures were properly aerated, large yields of oxalic acid were only obtained from hexoses, pentoses, and gluconic acid.

These facts lead to the conclusion that oxalic acid is probably formed by the oxidation of 2 end carbon atoms of molecules with at least 5 carbon atoms in the chain. On this view oxalic acid would be expected to arise from compounds of the types: CHO.CHOH.R, CH_2OH.CO.R, and COOH.CHOH.R, in which R contains at least 3 carbon atoms. Oxidation to a keto-acid, followed by hydrolysis, would result in the formation of oxalic acid + a compound RH:



The following series of reactions would account for the production of oxalic acid from glucose:



The first change of the above series is known to occur in *Aspergillus niger*, for Muller (1928) has isolated glucose-oxidase, which converts glucose into gluconic acid. In addition a parallel for the conversion of gluconic acid into fructuronic acid is afforded by the production of glucosone from glucose (Walker, 1932).

In our experiments with gluconic acid (Group 2 (*e*), p. 339) the production of 1 molecule of oxalic acid for every molecule of gluconic acid lost would have afforded strong evidence for the above scheme of oxalic acid formation. Such a ratio was not constantly obtained, but our results were easily explained by the further conversion of a proportion of the oxalic acid.

It is not maintained that the position of gluconic acid as an intermediate in the normal conversion of glucose into oxalic acid is definitely established, or that the reactions of this degradation exactly follow the scheme given above; but all the evidence points to a similar sequence of changes involving 2 adjacent end carbon atoms of the glucose molecule, and gluconic acid appears to be a likely member of the series.

Oxalic acid formation, however, is not to be looked upon simply as a series of straightforward chemical reactions, for the accumulation of oxalic acid is influenced by alteration of the external conditions. It appears that the rate of disappearance of oxalic acid is considerably accelerated by the presence of glucose (Group 4, Series *K*, p. 348). This aspect of the problem was not specially investigated, but it seems not unlikely that the oxidation of glucose is coupled in some way with the reactions involved in the disappearance of lactic and oxalic acids (and possibly other acids), so that the energy liberated by the breakdown of glucose supplies the energy necessary for the utilization of the acid.

The same group of experiments also revealed the existence of a reversible equilibrium between oxalic acid and some other substance, possibly glycogen. It has already been pointed out (Group 4, Series *K*, p. 348) that the oxalic acid concentration of a culture supplied only with glucose rises to the concentration attained by the loss of oxalic acid from cultures supplied with glucose + oxalic acid, and glucose + oxalic acid + lactic acid, after which the oxalic acid curves are strictly parallel (see Fig. 12). The presence of oxalic acid at a high concentration (about 1·3 %) in the solutions of glucose and glucose + lactic acid, almost completely suppressed the production of oxalic acid from glucose, for in neither case was any appreciable increase in oxalic acid content observed.

The above facts alone suggest that oxalic acid is in an equilibrium state, but further evidence of such a condition is supplied by the results of our experiments with pure glucose solutions. In several cases, one of which is described in detail in Group 3, p. 345, it was found that the oxalic acid concentration attained a fairly constant maximum

value, after which a slow disappearance of oxalic acid set in despite the presence of large quantities of sugar. These results are most readily explained by an equilibrium of the following nature:

$$\text{Glycogen} \rightleftharpoons \text{glucose} \rightleftharpoons \text{oxalic acid} \rightarrow CO_2.$$

It is experimentally found that when a glucose solution is supplied to a starved culture (see for example Fig. 9) large quantities of sugar disappear without any correspondingly large production of oxalic acid or CO_2. The excess glucose is probably converted into glycogen or some other reserve carbohydrate. Other glucose molecules are oxidized to oxalic acid, which rapidly accumulates; but in our view, with increase in the concentration of oxalic acid the forward change is arrested and the reverse charge change is accelerated, so that a condition of equilibrium arises. The slow fall in the equilibrium level with increasing age might be a result of a quickening of the backward reaction or a retarding of the forward reaction, or both causes might operate. A slight gradual decrease in the rate of conversion of glucose into oxalic acid is the most likely explanation, this view being supported by culture G_1, Group 3, p. 346, in which the rate of oxalic acid formation diminished as successive solutions of glucose were supplied.

If the above account be true, increase in the concentration of glucose beyond a certain point should have no effect on the equilibrium concentration of the oxalic acid. Similarly, if one supplies to the cultures a solution of glucose + oxalic acid with a higher oxalic acid concentration than the usual maximum concentration produced by the fungus, there should be a sharp fall to the equilibrium level. In our experiments, unfortunately, the concentration of oxalic acid in the glucose solutions supplied to the fungus was made approximately equal to the usual maximum oxalic acid concentration, so that no sharp fall in the quantity of oxalic acid was noticed. It has already been pointed out, however, that oxalic acid disappears even in the presence of large quantities of glucose. Further experiments, in which the cultures are to be supplied with varying concentrations of glucose only, and glucose + oxalic acid, should yield a decisive answer to this question.

The appearance of oxalic acid in starvation is in keeping with the above view. The low equilibrium level in starvation is explained by postulating that the conversion of glycogen into glucose is a slow process, and is hardly able to keep pace with the loss of oxalic acid as CO_2.

A further problem is presented by the effect of organic acids on this equilibrium. It was shown by Series K, Group 4, p. 348, that the

low oxalic acid concentration in the presence of lactic acid could not be referred to an acceleration of the rate of disappearance of the oxalic acid. In all cases investigated the effect of the acids was only temporary and oxalic acid was formed as soon as the acids had been used up. The suppression of oxalic acid accumulation was evidently due to an arrest of the forward reaction, either by direct slowing of the reaction velocity or by a side-tracking of an intermediate compound. The possibility is not lost sight of that the acid might be utilized for oxidation in preference to glucose, the accumulation of oxalic acid in this case depending upon the proportion of those quantities of the acid and glucose undergoing oxidation. Thus, in the case of lactic acid we might assume that a large amount of the acid and only a small quantity of glucose are oxidized, while with pyruvic acid a smaller proportion of the acid, and a larger proportion of glucose undergo oxidation, the result being that a much larger quantity of oxalic acid accumulates in the latter case. It is anticipated that further work will provide information as to the nature of this inhibiting effect.

SUMMARY

1. When solutions of oxalacetic acid were supplied to cultures of *Aspergillus niger*, which had been starved on distilled water for a few days, oxalic acid was not obtained, although the oxalacetic acid disappeared very rapidly.

2. Sodium oxalacetate was decomposed almost entirely into sodium pyruvate and sodium carbonate. The oxalic acid appearing after several days' exposure bore no quantitive relationship to the oxalacetic acid disappearing, and probably arose from the carbohydrate reserves of the mycelium.

3. It is therefore probable that oxalacetic acid is not an intermediate product in the formation of oxalic acid from glucose.

4. Oxalic acid did not appear when starved cultures were supplied with malic, succinic, and pyruvic acids, while Bennet-Clark & La Touche (1935) obtained similar results with citric and glycollic acids. In the present work, oxalic acid was not found when cultures were reared on solutions containing the sodium salts of fumaric, maleic, lactic, pyruvic and glycollic acids.

5. Of the acids investigated, only gluconic acid yielded oxalic acid when supplied in the free state to starving cultures. Oxalic acid was produced also from the hexoses glucose, fructose and galactose, and from the pentoses arabinose and xylose.

6. These facts indicate that oxalic acid arises by the oxidation of 2 end carbon atoms of a molecule with at least 5 atoms in the carbon chain. It is suggested that the first step in the reactions leading to the formation of oxalic acid is the production of a keto-acid. The "acid hydrolysis" of this compound would yield oxalic acid + a 3- or 4-carbon residue. In the case of glucose, the keto-acid is possibly fructuronic acid, with gluconic acid as its immediate precursor.

7. Evidence is given that oxalic acid is in reversible equilibrium with carbohydrate reserves of the mycelium.

8. It is shown that certain organic acids inhibit the accumulation of oxalic acid from glucose. Lactic acid has a striking effect, with pyruvic acid the effect is less marked, while malic acid and citric acid have little effect. It is found that oxalic acid attains its usual concentration when the concentration of the other acid has been greatly reduced. Glycollic acid is utilized so rapidly that the inhibiting effect is apparent only for a few days.

9. All our work points to the fact that the oxalic acid, which accumulates when cultures are reared on solutions containing salts of many organic acids, is a product of the carbohydrate reserves of the fungus, and is trapped by free base generated by the utilization of the organic anions. The absence of oxalic acid when certain acids are employed is referred to the inhibiting effect of the excess of those acids.

I wish to express my indebtedness to Professor T. A. Bennet-Clark for many helpful suggestions and friendly criticism.

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The Society for Experimental Biology holds three Conferences a year at which papers and demonstrations are given on various aspects of experimental zoology and botany. Short accounts of the botanical proceedings at these Conferences will appear in this *Journal*; and members of the Society are able to receive the *New Phytologist* on special terms. Further particulars of the activities of the Society may be obtained from the Secretaries: J. Z. Young, Magdalen College, Oxford, and E. Ashby, The University, Bristol.

FORTIETH CONFERENCE OF THE SOCIETY

The fortieth Conference was held at Leeds from 7 to 9 July 1937. Two sessions were of particular interest to botanists. At the first, papers were read on aspects of micro-structure in cells. Dr W. H. Pearsall was in the chair.

Mr F. A. Baker described how the disintegration of plant tissues by bacteria in the caecum of animals could be followed by microscopic observation. The micro-organisms produce in the cell wall zones of erosion, the direction of which is determined by the micellar structure of the wall. Loss of double refraction accompanies changed staining reactions in the zones of erosion. Dr R. D. Preston discussed the physical nature of the plant cell walls of three types of cell—*Valonia*, *Cladophora*, and conifer tracheids—as revealed by X-ray analysis. In *Valonia* and *Cladophora* the cellulose chains are arranged in two crossed spirals; in *Valonia* their arrangement corresponds with the striations on the wall visible under the microscope. In the tracheids of conifers there is a single spiral in the wall, and its angle of inclination to the axis of the wall decreases with age. The ratio: length of tracheid/cotangent of inclination of spiral is constant for the species. Professor F. G. Gregory and Dr K. M. Rudall took part in the discussion.

Dr Rudall read a paper on the arrangement of the protein chains in animal epidermal structures and showed how the pattern of the X-ray photograph changed according to the degree of extension of the chains.

Dr W. R. Atkin discussed how views on the structure of collagen and gelatin should be changed in accordance with the behaviour of these substances in titration curves on either side of the iso-electric point.

The second botanical session included papers on water relations and nitrogen metabolism in plants. Mr W. R. C. Handley described experiments on the effect of cooling the stem of a tree on the ascent of sap. If part of the stem is cooled to 2° C., starch migration and the formation of new vessels cease. If it is cooled to 0° C. the leaves above the cooled section wilt, and recover if the treatment is discontinued. Mr Handley emphasized that these observations are not in accordance with a purely physical interpretation of water movement in stems, and that living cells in the wood must be involved. He demonstrated that there was no obvious physical explanation of his data. Professor Bennet-Clark, and Messrs Boswell, Warne, and Preston, took part in the discussion.

Dr L. G. C. Warne gave data on some effects of potash deficiency on water relations. He showed that in the absence of potassium the leaf area of beet and sorghum was reduced, and that this reduction was entirely referable to a reduction in cell size; as a consequence the stomatal frequency is increased and transpiration is greater. Absence of potassium reduces also the conductivity of the stem to water. Messrs Pearsall, Boswell, and Ashby took part in the discussion.

Dr W. H. Pearsall read a paper on protein synthesis in leaves. His data showed that protein synthesis occurs only in the younger parts of daffodil leaves, while hydrolysis takes place in the older parts. There was evidence of an effect of light on protein synthesis. From nitrogen 'balance sheets' it appeared that not all the nitrogen taken in by a plant is utilized; some is released as gaseous nitrogen owing to the combination of amino-nitrogen with nitrous acid in the plant. Mr Noltie and Dr Archbold took part in the discussion.

Messrs Bengry and Pearsall read a paper on the growth of pure cultures of *Chlorella*. They pointed out that the growth is exponential until there is a concentration of 6000 individuals per cubic mm., and that growth finally ceased when the concentration was 50,000 per cubic mm. If glucose is added to the medium cultures will grow exponentially, even in the dark. The precise effect of light in increasing the growth rate was discussed by Messrs Boswell, Hatcher, and Ashby.

Among the demonstrations of interest to botanists were preparations of macerated sieve tubes and vessel endings, shown by Dr L. I. Scott and Professor J. H. Priestley; photographs and graphs illustrating the quantitative morphological development of *Lemna minor*, by Mr E. J. Winter, and exhibits illustrating the various papers.

The next Conference of the Society will be held in London on 20–22 December 1937.

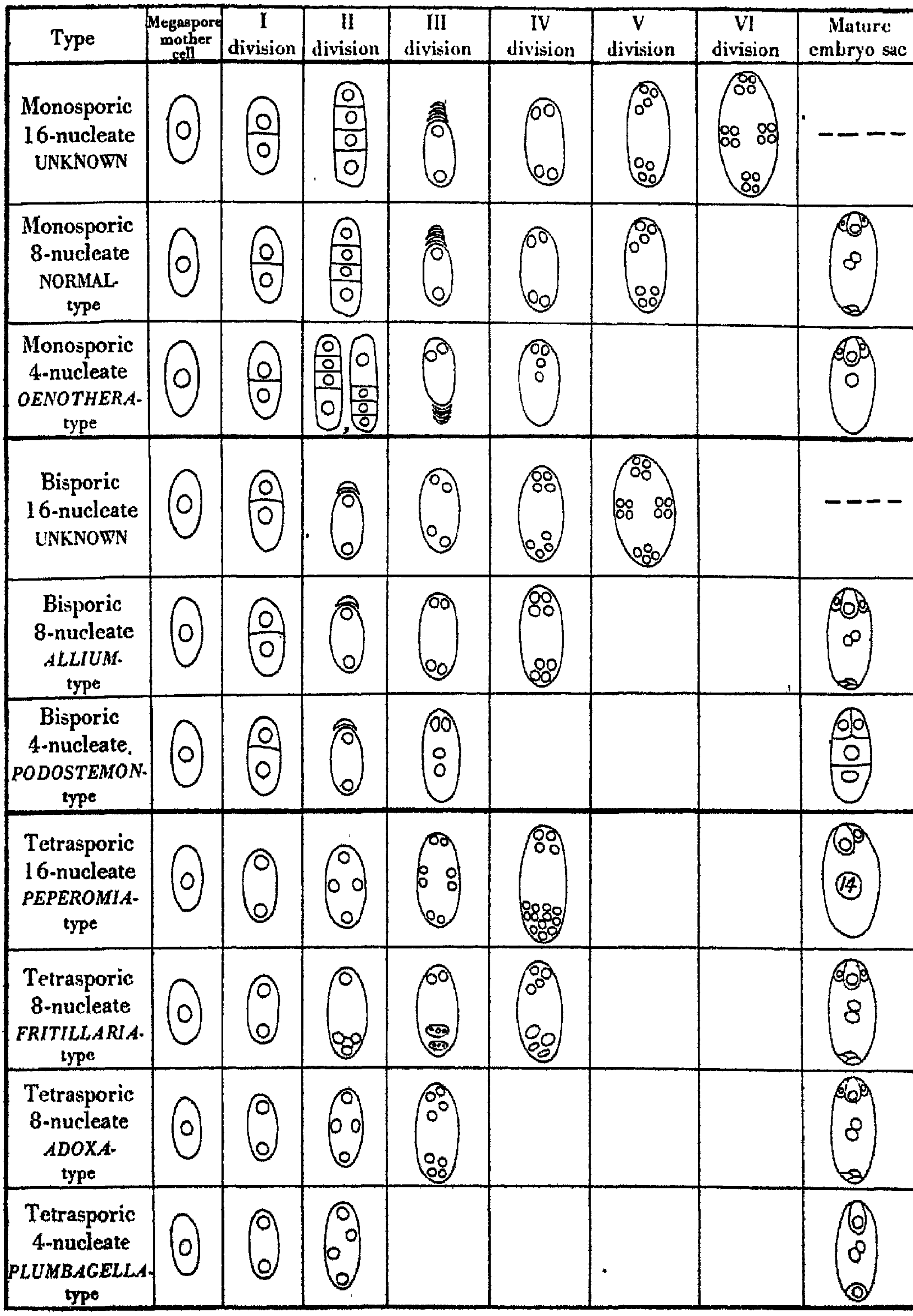


Fig. 1. Types of embryo sac development in angiosperms.

(4) *4-nucleate*; there are only two divisions and the megaspore nuclei themselves enter into the organization of the embryo sac (*Plumbagella*-type).

In the following pages each of these types will be discussed in fair detail.

MONOSPORIC; 16-NUCLEATE

Monosporic embryo sacs with more than eight nuclei are rare. Such a condition may be due to the following reasons and the cause should in each case receive careful study:

(1) Degeneration of nucellar cells and the passing in of their nuclei into the embryo sac, as reported by Quisumbing & Juliano (1927) in *Cocos nucifera*. This is comparable to the situation in many gymnosperms where the nuclei of the jacket cells wander inside the egg, but the same appearance may also be brought about due to inadequacies of technique.

(2) Fusion of two embryo sacs lying adjacent to one another. The 16-nucleate embryo sac of *Elatine hydropiper* seen by Frisendahl (1927, Fig. 32), consisting of two egg apparatuses, two pairs of polar nuclei and two groups of three antipodals each, is clearly derived by this method. Oksijuk (1929) found some exactly similar cases in *Reseda alba*, and has very recently (1935) reported the same thing in *R. inodora*. When one of the embryo sacs is at a younger stage of development than the other, the "compound embryo sac"[1] formed by their fusion may have less than sixteen nuclei.

(3) A sixth division of some or all of the nuclei of the embryo sac. Usually such an abnormality is due to irregularities caused by a hybrid origin of the species under investigation (for literature see Chiarugi & Francini (1930) on *Ochna serrulata*; and Steinegger (1933) on apples).

Dessiatoff (1911) reported that in *Euphorbia virgata* there was a monosporic embryo sac with sixteen nuclei, but Modilewski (1911) demonstrated that this was of the *Peperomia*-type. Mauritzon (1933, p. 35) reports that in *Crassula Schmidtii* and *Umbilicus intermedius* there is a fourth division of the megaspore nucleus resulting in

[1] Juliano (1934) figures a peculiar embryo sac in a fallen flower of *Sandoricum Koetjape*. This has a normal egg apparatus and two polar nuclei. In the chalazal region there is a large cytoplasmic vesicle extending up to the middle of the sac. This was found to contain fourteen nuclei. The author believes that this could not be antipodal in origin, since the antipodals are very ephemeral in this species. It is considered probable that the embryo sac itself was formed from the third megaspore and the vesicle from free nuclear divisions in the fourth.

a 16-nucleate embryo sac with four synergids, two egg-like cells, six antipodals and four free nuclei. Cases like these are of great interest, but we need more precise information about their origin and the frequency with which they occur in the plants in question. For the present we may say that a monosporic embryo sac of the 16-nucleate type is unknown to-day except as an abnormality.

NORMAL-TYPE

Eight-nucleate embryo sacs formed from the divisions of a single megaspore are remarkably uniform in their development. The abnormalities may be grouped under three headings: *tetrad formation*,

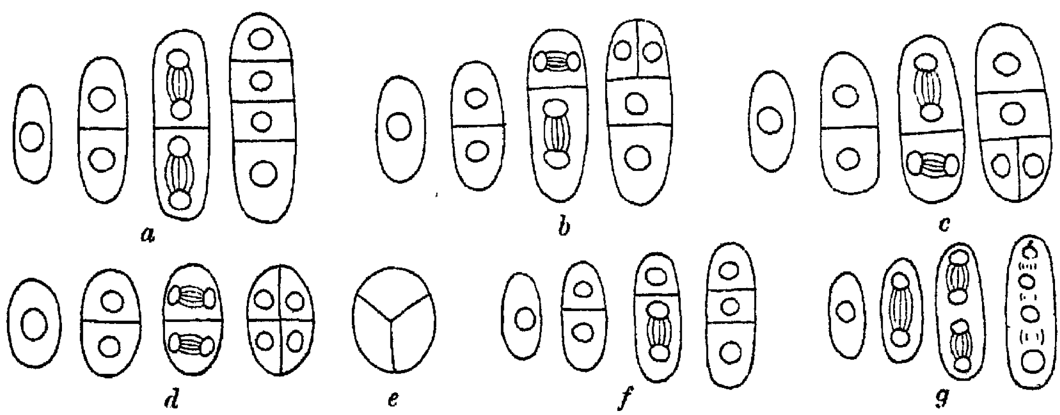


Fig. 2. Types of megaspore tetrads: (*a*) linear; (*b*) T-shaped; (*c*) ⊥-shaped; (*d*) isobilateral; (*e*) tetrahedral; (*f*) row of three cells of which the uppermost is a dyad cell; (*g*) four megaspore nuclei unseparated by walls.

divisions of the megaspore nucleus and *organization of the mature embryo sac*.

Tetrad formation.[1] In the majority of cases the megaspores are arranged in a linear row, and tetrads of this type are consequently called "linear" (Fig. 2*a*), but T-shaped tetrads[2] are also very common (Fig. 2*b*). A row of three cells is also frequently met with, but in about half the cases this is likely to be a T-shaped tetrad, one of whose megaspores is to be looked for in an adjacent section. In other cases (Fig. 2*f*) where we have really only three cells, one is not a megaspore but a dyad cell that has failed to divide again. Such an appearance has often been wrongly described in literature as a row of "three megaspores". Isobilateral tetrads (Fig. 2*d*) are known only

[1] An interesting situation has been reported in two Crassulaceae, *Rosularia pallida* and *Sedum sempervivoides* (Mauritzon, 1933, p. 25), in which the megaspores elongate and become haustorial.

[2] Salisbury (1931, p. 561; see also Pl. XVIII, fig. 3) has mistaken the upper two megaspores of a T-shaped tetrad of *Ranunculus parviflorus* as wall cells (see criticism by Singh, 1936, p. 85).

as abnormalities, as for instance in *Myrtus communis* (Greco, 1930) and *Urginea indica* (Capoor, 1937*a*). A tetrahedral arrangement (Fig. 2*e*) is still more rare and was noted in recent literature only in *Nymphoides peltatum* (Stover, 1932). ⊥-shaped tetrads (Fig. 2*c*) occur occasionally in the Onagraceae (see under *Oenothera*-type), and may be expected only in such cases where the uppermost megaspore functions. Finally there are also such cases where the four nuclei are arranged in a linear row but without any cell plates separating them (Fig. 2*g*).

Divisions of the megaspore nucleus. In the vast majority of angiosperms it is only the chalazal megaspore that grows further, while the other three are suppressed and soon degenerate. There are, however, a good many exceptions on record (see Schnarf, 1929, pp. 96–120). In the genus *Rosa* (Hurst, 1931) it is constantly the micropylar megaspore that functions, and in *Aristotelia racemosa* it is always the third from the micropylar end (Mauritzon, 1934*b*). Sometimes two megaspores of a tetrad begin to grow simultaneously (Nast, 1935, in *Juglans regia*), but usually only one of the sister embryo sacs reaches maturity.

The functioning megaspore stage is characterized by a beginning of vacuolation. At first there are two vacuoles, one on each side of the nucleus in the long axis of the cell. After the first division, the two daughter nuclei migrate to the poles and a large vacuole appears in the centre. The subsequent divisions are rapid and simultaneous and result in a grouping of four nuclei at each end.[1]

Departures in the sequence of these divisions are usually in the nature of abnormalities that may occur now and then in any plant and have no special significance. Kuhn (1928, p. 420) found that in *Thalictrum Fendleri* the last division (after the 4-nucleate stage) does not always proceed at the same rate in all the four nuclei. Sabet (1931) has figured a similar abnormality in *Calotropis procera*. Millsaps (1936) reports that in *Paulownia tomentosa* it is usual for the primary chalazal nucleus to divide a little earlier than the micropylar so that a 3-nucleate embryo sac is formed for a short time. This is rather rare because delayed division commonly occurs in the lower part of the embryo sac.

Organization of the mature embryo sac. Most of the abnormalities concern the antipodal cells whose early disappearance has been recorded in many angiosperms and is a characteristic feature of

[1] Sabet (1931) figures an abnormal embryo sac of *Calotropis procera* in which these two groups are arranged like pollen tetrads.

some genera and even families. Such is the case in almost all of the Lythraceae so far investigated (Mauritzon, 1934*a*; Joshi & Venkateswarlu, 1936*a*), and this points the way for the derivation of the *Oenothera*-type of embryo sac, in which the antipodals are altogether absent.

Among other plants in which an early degeneration of the antipodals has been recorded, we may specially mention the following names: *Petunia violacea*, in which Cuchtmanova (1930) reports a "dégénération très prompte" of the nuclei even before the organization of the egg apparatus; *Trianthema monogyna* (Bhargava, 1935), where not only the antipodals but also the synergids degenerate very early so that the mature embryo sac has only two nuclei, i.e. the egg and secondary nucleus; and *Duabanga sonneratioides* (Venkateswarlu, 1936).

The early disappearance of the antipodals has misled some authors in the past, and it seems certain that a good many reports of 5-nucleate embryo sacs (before polar fusion) and 4-nucleate embryo sacs (after polar fusion) are due to misinterpretations caused by inadequacy of material of the right age. Puri (1934) has shown that the 5-nucleate embryo sac recorded by Rutgers (1923) in *Moringa oleifera* is really 8-nucleate. The same may be said with some confidence of *Linaria vulgaris*, *Antirrhinum majus*, *Melampyrum silvaticum*, *M. pratense* and *Tozzia alpina* (Schmid, 1906),[1] *Garcinia Kydii* and *G. Treubii*[2] (Treub, 1911), *Monophyllaea Horsfeldii* (Oehlkers, 1923), *Chamaedorea concolor* (Suessenguth, 1921), *Sisyrinchium anceps* (Haeckel,[3] 1930, p. 73) and *Linaria genistaefolia* (Persidsky, 1934). In the last-named instance the author says that the antipodals are "apparently" not formed at all.

On the other hand, there are also cases in which a genuine reduction has occurred in the number of nuclei at the chalazal end of the embryo sac. Thus Sharp (1912) found only 6-nucleate embryo sacs in *Phajus grandifolius*, *Corallorhiza maculata* and *Broughtonia sanguinea*—a condition due to the primary chalazal nucleus dividing only once after the 2-nucleate stage.[4] In *Cinnamomum camphora* (Giuliani,

[1] The author writes (p. 271): "Zunächst gibt es eine Anzahl von Scrophulariaceae bei denen Antipoden überhaupt nicht angelegt zu werden scheinen, wenigstens gelang es mir nie, das Vorhandensein solcher zu konstatieren."

[2] Mr V. Puri of Meerut finds that *Garcinia livingstonii* has the normal eight nuclei in the embryo sac.

[3] Since this was written, Miss Haeckel has told me, during the course of a personal talk at Halle/S., that after the publication of her work she was able to find the antipodals in her slides, although they are very small and ephemeral.

[4] Svensson (1928) and Reeves (1930) report that such is also the case sometimes in *Limosella aquatica* and *Medicago sativa* respectively.

1928) it seems probable that the primary chalazal nucleus does not divide at all, so that the embryo sacs are only 5-nucleate; in later stages when this also has disappeared only four can be seen.

The opposite condition, i.e. an increase in the number of antipodal cells, is also of frequent occurrence. In the Gramineae and Compositae it seems to be almost universal. *Sasa paniculata* (Yamaura, 1933) perhaps represents the most extreme case with as many as 300 antipodal cells.

Abnormalities concerning the synergids are much rarer. Rodolico (1930) reports that they are specially prominent in *Buphthalmum salicifolium*, and Paetow (1931) writes that in *Dysoxylum ramifolium* they reach almost to the middle of the embryo sac. Joshi & Venkateswarlu (1935) reported that in *Ammania baccifera* the two synergids fuse to form a syn-synergid which becomes multi-nucleate and surrounds the embryo like a collar. Later, the same authors (1936*b*) found that what they had been looking at was a mass of endosperm nuclei at the micropylar end of the sac.[1]

Cases where antipodal cells have assumed an egg-like appearance have been frequently recorded, but sometimes the whole embryo sac shows an inversed polarity, i.e. the egg apparatus lies in the chalazal part and the antipodal cells in the micropylar part. Dahlgren (1927, pp. 210–11) gave a list of such cases, and to these may be added *Saccharum officinarum* (Dutta & Subba Rao, 1933) and *Woodfordia floribunda* (Joshi & Venkateswarlu,[2] 1935*b*).

More interesting than any of these are two saprophytic Gentianaceae, *Leiphaimos* sp. and *Cotylanthera tenuis*,[3] investigated by Oehler (1927). The ovules are here devoid of any integument and the embryo sac is completely inverted, i.e. the antipodal cells are on the side towards the nucellar epidermis and the egg apparatus towards the funiculus. This is of particular significance, since it shows that "die Samenanlagen von *Leiphaimos* und *Cotylanthera* sind nur äusserlich orthotrop, innerlich aber anatrop d.h., ihr Embryosack ist wie in anatropen Samenanlagen orientiert".

[1] Great care is needed in such interpretations. Rutgers (1923) reported that there is a free nuclear egg with sixteen nuclei in *Moringa oleifera*, while Puri (1934) has now shown that the first division of the zygote is followed by wall formation as in other angiosperms. It seems certain that Rutgers was looking at endosperm nuclei, while the egg itself entirely escaped his notice in earlier stages!

[2] Joshi & Venkateswarlu (1935*b*, p. 843) write that theirs is the "first" clear case of reversed polarity observed in an 8-nucleate embryo sac. Actually, however, the best instances of this kind are to be seen in the Loranthaceae, Balanophoraceae, and *Leiphaimos* (Oehler, 1927).

[3] This plant is apogamous.

A brief reference may finally be made to the tubular embryo sacs of *Siparuna Eggersii* (Heilborn, 1931). The megaspore mother cell gives rise to the usual tetrad of megaspores of which the chalazal functions and the rest degenerate. This elongates considerably and penetrates downward into the nucellus but soon encounters a hypostase which checks further development. Here it becomes coiled into several turns and the end swells into a vesicle, which finally bursts and releases its contents into a nucellar cavity that has formed in the meantime just above the hypostasis. Although fertilization does not occur and the embryo sac soon degenerates, we have here an illustration of the homologies between the mega- and microspores of angiosperms; for just as pollen grains may, under certain circumstances, attain the form of embryo sacs (Stow, 1930), so embryo sacs can sometimes grow like pollen tubes.

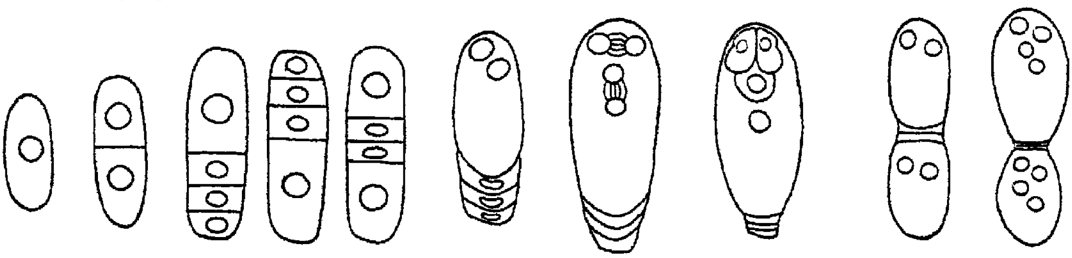
In several instances (*Orobanche Hederae* (Glišić, 1929); *Newcastlia insignis* and *Congea villosa* (Junell, 1934); *Utricularia coerulea* (Kausik, 1935); *Putoria calabrica* (Fagerlind, 1936); and others) embryo sacs are known to protrude out of the micropyle into the loculus of the ovary, but the condition reported by Rauch (1936) in *Scurrula atropurpurea* and *Dendrophthoe pentandra* is without a parallel. Ovules and integuments are absent as in the other Loranthaceae. The embryo sac undergoes a remarkable elongation both towards the top and the bottom; at the chalazal end it is soon stopped by a layer of collenchymatous cells, but the tip grows out halfway upward into the conducting tissue of the style! Fertilization occurs here by the incoming pollen tubes, and the embryos are thrust down again by the elongating suspensors![1]

Oenothera-type

Geerts (1908) was the first to publish a clear account of the development of the embryo sac in *Oenothera lamarckiana* and Modilewski (1909*b*) described the same features in *O. biennis*, *Epilobium angustifolium*, *E. dodonaei* and *Circaea lutetiana*. Only four divisions intervene between the megaspore mother cell and the egg, and the first two of these are the reduction divisions. Contrary to the normal condition, it is usually the micropylar megaspore that functions here (Fig. 3*c*), but sometimes it is the chalazal (Fig. 3*d*), and occasionally both of them may start to form embryo sacs (Fig. 3 *e*, *i*, *j*). In *Anogra pallida* (Johansen, 1931*c*), *Zauschneria*

[1] Such a condition recalls that in *Welwitschia*, but of course the two plants are too far apart to permit further comparisons.

latifolia (Johansen, 1931*b*) and *Ludwigia parviflora* (Maheshwari & Gupta, 1934), ⊥-shaped tetrads (Fig. 2*c*) have sometimes been observed. In every case the megaspore nucleus undergoes only two more divisions, and all the four nuclei so formed lie in the micropylar end of the embryo sac. Three of these organize into the egg apparatus and the fourth functions as the single polar nucleus. The third mitosis is entirely omitted, and consequently there are no antipodals nor a lower polar nucleus. It happens, however, that the three degenerating megaspores persist for a long time at the base of the embryo sac and may give the deceptive appearance of antipodal cells (Fig. 3*h*).



a *b* *c* *d* *e* *f* *g* *h* *i* *j*

Fig. 3. Stages in development of *Oenothera*-type of embryo sac: (*a*) megaspore mother cell; (*b*) dyad; (*c, d, e*) tetrads of megaspores; (*f, g*) 2- and 4-nucleate stages; (*h*) mature embryo sac; (*i, j*) both micropylar and chalazal megaspores growing into embryo sacs.

This type of development has been reported in all the members of the Oenotheraceae so far investigated, and may indeed be regarded as a valuable diagnostic character for the family. The only exception is *Trapa*, which has an 8-nucleate embryo sac, but even here the antipodal nuclei acquire no walls and promptly degenerate. Ishikawa (1918), who gave a brief account of the development in *T. natans*, thinks that the difference is important enough to justify the separation of this genus from the family.[1]

The following list attempts to bring together all the species of this family that have been investigated and found to have tetranucleate embryo sacs:

Name of plant	Author
Anogra pallida	Johansen (1931*c*)
Boisduvalia densiflora	Täckholm (1915)
Circaea lutetiana	Modilewski (1909*b*), Werner (1915)
C. pacifica	Johansen (1934)
C. quadrisulcata	Ishikawa (1918)
Clarkia sp.	Werner (1915)
C. elegans	Täckholm (1915), Johansen (1930*a*)
C. pulchella	Täckholm (1915)
Epilobium angustifolium[2]	Modilewski (1909*b*), Werner (1915), Täckholm (1915), Ishikawa (1918)

[1] In the latest edition of the *Syllabus der Pflanzenfamilien* the genus *Trapa* has been placed in the family Hydrocaryaceae (see Engler, 1936, p. 306).

[2] Michaelis (1925) has also investigated some species of *Epilobium*, but his account deals chiefly with the development of the embryo and chromosome numbers.

Name of plant	Author
Epilobium Dodonaei	Modilewski (1909*b*)
E. hirsutum	Täckholm (1915)
E. hirsutum × *E. montanum*	Håkansson (1924)
E. parviflorum	Schwemmle (1924)
E. roseum	,,
Fuchsia sp.	Werner (1915)
F. coccinea	Täckholm (1915)
F. "Emile de Wildeman"	,,
F. fulgens	,,
F. "Marinka" and other cultivated varieties	,,
F. procumbens	,,
F. pumila	,,
Gaura Lindheimeri	Ishikawa (1918)
G. parviflora	,,
Gayophytum ramoisissimum	Johansen (1932)
Godetia sp.	Ishikawa (1918)
G. amoena	Täckholm (1915)
G. "gloriosa"	,,
G. Whitneyi	,,
Hartmannia tetraptera	Johansen (1929)
Jussieua repens	Ishikawa (1918), Maheshwari & Gupta (1934)
J. suffruticosa	Täckholm (1915)
J. villosa	,,
Lopezia coronata	Täckholm (1914)
L. parviflora	Maheshwari & Gupta (1934)
L. prostrata	Ishikawa (1918)
Oenothera albata	Hoeppener & Renner (1929)
O. biennis	Modilewski (1909*b*), Davis (1910), Renner (1914), Werner (1915)
O. coccinea	Werner (1915)
O. cruciata	Rudloff & Schmidt (1932)
O. fallax	Langendorf (1930)
O. grandiflora	Gerhard (1929)
O. Hookeri	Rudloff & Schmidt (1932)
O. Hookeri albata	Langendorf (1930)
O. Lamarckiana	Geerts (1908), Werner (1915), Haberlandt (1927)
O. "Lamarckiana-gigas"	Hoeppener & Renner (1929)
O. "R.-Lamarckiana"	Rudloff & Schmidt (1932)
O. lutescens	Hoeppener & Renner (1929)
O. muricata	Haberlandt (1927)
O. "R. muricata"	Rudloff & Schmidt (1932)
O. nutans	Ishikawa (1918)
O. pachycarpa	Rudloff (1930)
O. pycnocarpa	Ishikawa (1918)
O. rhizocarpa	Werner (1915)
O. rubiflava	Hoeppener & Renner (1929)
O. rubrinervis	O'Neal (1923), Rudloff & Schmidt (1932)
O. rubririgida	Langendorf (1930)
O. suaveolens	Hoeppener & Renner (1929)
O. tetraptera	Werner (1915)
Stenosiphon linifolium	Johansen (1931*c*)
Taraxia ovata	Johansen (1931*a*)
Zauschneria latifolia	Johansen (1931*b*)

Abnormalities with regard to the number of nuclei are relatively rare. When less than four occur, this is mostly due to a failure of division of the primary synergid nucleus resulting in a 3-nucleate embryo sac with only one synergid, an egg and a polar nucleus (as in *Hartmannia tetraptera*, Johansen, 1929). A multinucleate

condition often results due to a fusion of two gametophytes. As mentioned before, there is a tendency in several members of this family for a concurrent development of both the terminal megaspores, thus resulting in twin embryo sacs (Fig. 3*j*). The two megaspores lying in the middle are soon crushed, and if the separating wall dissolves, eight (four from each gametophyte) or six (four from one and two from the other) nuclei may be seen in a common chamber.

Täckholm (1915, p. 352) figures an embryo sac of *Fuchsia procumbens* which must have originated from a 7-nucleate condition; the three cells of the egg apparatus are all binucleate and there is a single polar nucleus. The same author also described some embryo sacs with five nuclei in *Jussieua, Godetia* and *Fuchsia*, and believes that the extra nucleus probably arose by a division of the polar nucleus.

Haberlandt (1927) records several interesting abnormalities in *Oenothera lamarckiana* and *O. muricata*, and cites cases where the number of nuclei may ascend up to fifteen, a condition resulting in his opinion from a "Rückbildung" or "Vegetativwerden des Embryosackes". A still more striking and perhaps unparalleled situation exists in *Anogra pallida* (Johansen, 1931*c*), in which the number of nuclei in some well-nourished embryo sacs may be as high as 140. This is due to a repeated amitotic division of the polar nucleus which seems to possess an unusual vitality in this plant.[1]

We may now turn to some other cases where an *Oenothera*-type of embryo sac has been reported. Arnoldi (1912) stated that *Ceramanthus, Glochidion* and *Codiaeum* also belong here. For *Codiaeum* (p. 143) he writes: "Die Urmutterzelle des Embryosackes von *Codiaeum* wird durch eine gewöhnliche Reduktionsteilung in eine in Fig. 8 abgebildete Tetrade zerlegt. Die unterste Zelle wird zum Embryosack, in dem durch zweimalige Kernteilung vier Kerne entstehen (Figs. 9–11). Damit werden die Teilungen eingestellt. Der Embryosack führt also ausser der Eizelle und den zwei Synergiden nur noch einen Kern (Fig. 12). Weder der andere Polkern, noch die antipodalen Kerne lassen sich auf der durch den Embryosack geführten Schnittserie entdecken."

The term "*Codiaeum*-type" thus came to be regarded as synonymous with "*Oenothera*-type", and some authors have actually preferred the former (Palm, 1915; Wettstein, 1935). A recent investigation of *Codiaeum variegatum* (Lundberg, 1931) has, however, shown that the embryo sac is really 8-nucleate and arises in the normal way. Arnoldi's error is explained by an early fusion of the polar

[1] This may also be regarded as the formation of a haploid parthenogenetic endosperm.

nuclei and a degeneration of the antipodals. Maheshwari & Chowdry (1937) have shown the same thing in *Phyllanthus* (=*Ceramanthus*) *niruri*, and it seems certain that *Glochidion* will yield similar results. Thus the occurrence of the *Oenothera*-type of embryo sac in the Euphorbiaceae remains unproved and the name "*Codiaeum*-type" has no meaning.

We may now pass on to *Gastrodia elata* (Kusano, 1915), in which the megaspore mother cell forms a row of three cells of which the chalazal functions. The 2- and 4-nucleate stages follow. According to the author there are no further divisions, and an egg apparatus and a single polar nucleus are now organized. As in *Cypripedium* (Pace, 1907), a synergid nucleus is said to leave its place and take part in triple fusion. It will be shown later than Pace's account of *Cypripedium* was incorrect and the same may be said of *Gastrodia*.

Recently Miss Parks (1935) has reported a 4-nucleate embryo sac in *Commelinantia Pringlei* and *C. anomala*, said to arise from the lowest cell of a row of three (as in *Gastrodia*). *Commelina benghalensis* (Maheshwari & Singh, 1934), a closely allied plant, is perfectly normal. It seems certain that the antipodal nuclei *are* formed in Miss Parks' plants also, but were overlooked by her due to their ephemeral nature.

Bisporic; 16-nucleate

Bisporic embryo sacs with sixteen nuclei are not known with certainty in any plant. Campbell (1910, 1911) investigated *Pandanus artocarpus*, *P. odoratissimus* and *P. coronatus* and reports many-nucleate embryo sacs in each case. Schnarf (1929, p. 197) has provisionally included *Pandanus* under bisporic embryo sacs, but Campbell's account is very old and lacks clearness. It is very desirable that this genus be reinvestigated in detail.

Modilewski (1929, p. 38) writes: "Ich habe einmal statt eines achtkernigen einen sechzehnkernigen Embryosack, wo die Kerne, ohne Zellen auszubilden, parallel der Längsaxe des Embryosackes lagerten, beobachtet. Falls die Art, in welcher dieser Embryosack entstanden war, zu dem *Scilla*-Typus gehörte, wie es bei normalen Embryosäcken von *Allium odorum* geschieht, so haben wir hier ein Beispiel eines bisporischen sechzehnkernigen Embryosackes."

Allium-type[1]

A bisporic embryo sac with eight nuclei was first reported by Strasburger (1879) in *Allium fistulosum*, and has been described since then in many angiosperms belonging to different families.

[1] Most authors have used the name "*Scilla*-type", but this seems to be less appropriate since *Allium* has the claim of priority.

The megaspore mother cell divides into two cells of which the upper usually degenerates quickly while the lower undergoes three divisions to form an 8-nucleate embryo sac. There is thus a total of four divisions—one less than in the Normal-type.

In some cases the nucleus of the upper dyad cell also divides, but degeneration may start before the division is completed (*Lycopsis arvensis*, Svensson, 1925); in other cases the nuclear division is followed by an anticlinal or periclinal wall. In a few plants the

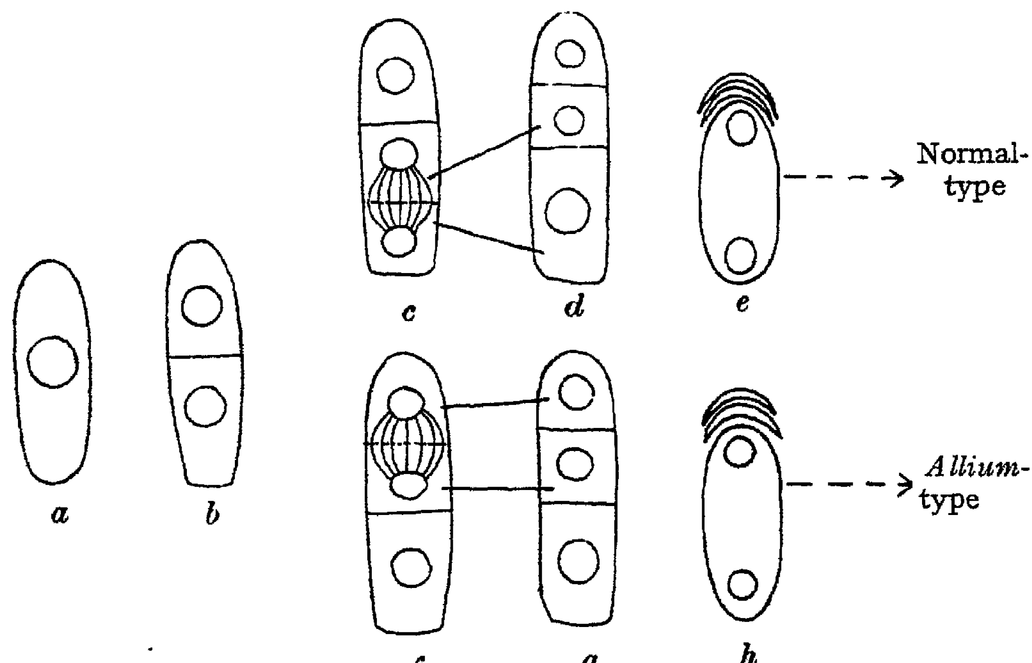


Fig. 4. Explanation of origin of "row of three cells": (*a*) megaspore mother cell; (*b*) dyad; (*c, d, e*) only the lower dyad cell has divided. Of the three cells in (*d*) the chalazal cell develops into an embryo sac (Normal-type); (*f, g, h*) only the upper dyad cell has divided. Of the three cells in (*g*), the chalazal develops into an embryo sac (*Allium*-type).

nucleus may undergo more than one division, and finally there are some cases where it is the upper dyad that functions and the lower degenerates. In *Scilla nonscripta* (Hoare, 1934) the micropylar functions, but the nucleus of the chalazal cell also divides and forms an "antigone" consisting of four nuclei.

Finally, it is necessary to call attention to the "row of three cells", which has led to some confused interpretations. This may arise in two ways: either the uppermost cell is an undivided dyad in which case the development is to be regarded as of the normal monosporic type (Fig. 4 *c, d, e*), or the lower cell is an undivided dyad, in which case the development is to be regarded as of the bisporic type (Fig. 4 *f, g, h*). Shadowsky fails to make this point clear in his papers on *Pancratium maritimum* (1925*a*) and *Pistia stratioites* (1931), and

his figures admit both interpretations. The occurrence of the *Allium*-type remains unproved in such cases.

As described under the Normal-type, we have here also the occurrence of egg-like antipodal cells in some plants. Modilewski (1931) reports that in *Allium nigrum* one antipodal cell frequently resembles an egg cell, while the other two closely resemble the synergids even in the possession of a filiform apparatus. Messeri (1931) has reported the occurrence of such "antipodal egg cells" in *A. subhirsutum* and *A. schoenoprasum.* Rutishauser (1935) has recently reported in *Korthalsella Dacrydii* a condition somewhat similar to that already described in *Leiphaimos* (Oehler, 1927). Of the two cells formed from the megaspore mother cell, the lower degenerates and the upper functions. After the 4-nucleate stage the embryo sac begins to curve round at the base and soon attains a U-shaped form. The two nuclei at each end now undergo the last division resulting in the 8-nucleate stage. The peculiar thing is that the egg apparatus differentiates at the morphologically lower end of the sac and the antipodals at the upper end. "Der Embryosack verhält sich so, *als ob er einer anatropen Samenanlage angehören würde.* Da wir kein Organ entdecken können, das einer anatropen Samenanlage entspricht, wollen wir den Embryosack von *K. Dacrydii* als in sich anatrop bezeichnen" (Rutishauser, p. 407). Such embryo sacs, anatropous in themselves, seem to be characteristic of other Loranthaceae as well, although their earlier development does not always seem to agree with that described for *Korthalsella.*

There are a number of plants under the *Allium*-type, in which the embryo sacs have less than eight nuclei (see Fig. 5). Reduction always occurs at the chalazal end. The Alismaceae, investigated by Johri (1935*a*, *b*, *c*, 1936*b*), are specially interesting in this connexion. In *Limnophyton obtusifolium* (Johri, 1935*a*) the embryo sacs have sometimes all eight nuclei formed in the usual way, more often there are only seven (this is due to a failure of division of the lowest nucleus of the 4-nucleate stage) but the commonest of all is the 6-nucleate condition. The development proceeds normally up to the 4-nucleate stage, but after this only the micropylar pair divides while the chalazal remains undivided.

Further reduction has been seen in *Nipa fruticans* (Radermacher, 1925), *Echinodorus ranunculoides* (Dahlgren, 1928), *E. macrophyllus* (Dahlgren, 1934), *Butomopsis lanceolata* (Johri, 1936*a*) and several genera of the Podostemonaceae, viz. *Apinagia, Cladopus, Lophogyne, Mourera, Oenone, Rhyncolacis, Tristicha* (Went, 1910, 1912, 1926)

and *Lawia* (Magnus, 1913). In these plants the primary chalazal nucleus does not divide at all, and the embryo sacs are only 5-nucleate with a normally organized egg apparatus, a single polar nucleus and one degenerated nucleus at the chalazal end. In *Lawia* this sometimes remains healthy for a longer time and may even fuse with the upper polar nucleus (Magnus, 1913).

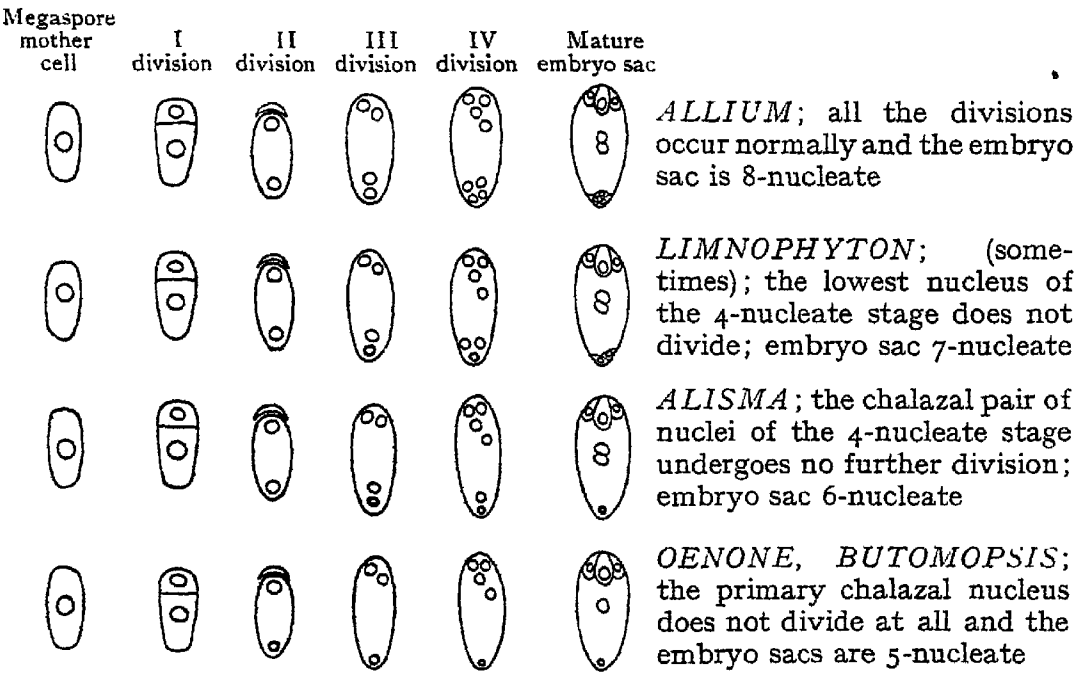


Fig. 5. Diagrams to show reduction of number of nuclei in chalazal part of embryo sac of *Allium*-type.

Pace (1907) had described a peculiar type of embryo sac in *Cypripedium spectabile*, *C. pariflorus*, *C. pubescens* and *C. candidum*, in which the lower dyad cell was reported to divide twice, resulting in a pair of nuclei at each end of the embryo sac. The upper pair formed the two synergids and the lower contributed to the egg and single-polar nucleus. At the time of fertilization one of the synergid nuclei was observed to descend and take part in triple fusion. This type of development, so far known as the *Cypripedium*-type (Palm, 1915), was subjected to many criticisms, and a recent investigation of *C. guttatum* (Prosina, 1930) and some other species of this genus (Francini, 1931) shows that the development is essentially the same as in *Allium* with some reduction at the chalazal end, so that frequently there are only six or five nuclei in the embryo sac. A 4-nucleate condition may arise only in very exceptional cases, but even here its origin is quite different from that described by Pace (see Francini, 1931).

The following is a list of such angiosperms in which the *Allium*-type has been reported so far. When any name is preceded by an asterisk, this indicates doubt about the reliability of the observations.

When there are two such marks, this either means that such an interpretation was later found to be wholly incorrect or that there are very strong reasons to think that this is so. "s" indicates that the development is usually of the normal type, but *Allium*-type was observed in isolated cases.

	SAURURACEAE	
Saururus cernuus	Johnson (1900*a*)	—
	SALICACEAE	
Salix petiolaris *S. glaucophylla* *S. discolor* *S. tristis* *S. cordata*	Chamberlain (1897)	Sometimes *Adoxa*-type is also reported to have been found, and the author states that such variations are common in the genus. Jönsson (1879–80), however, found Normal-type in *S. fragilis* and *S. aurita*, and Håkansson (1929) has done the same in *S. viminalis* and *S. caprea*
**Populus canadensis* **P. tremula*	Graf (1921)	The author is himself somewhat doubtful
	PODOSTEMONACEAE	
Apinagia divertens	Went (1910)	In all these cases the embryo sacs are only 5-nucleate, due to a reduction in the number of divisions at the chalazal end
A. perpusilla	Went (1910, 1926)	
Cladopus Nymani	Went (1926)	
Lawia zeylanica	Magnus (1913)	
Lophogyne capillacea	Went (1910)	
Mourera fluviatilis	Went (1910, 1926)	
Oenone Imthurni	Went (1910)	
O. Richardiana	Went (1926)	
O. versteegiana	Went (1910, 1926)	
Rhyncolacis macrocarpa	Went (1912)	—
Tristicha hypnoides	Went (1926)	—
	LORANTHACEAE	
**Arceuthobium americanum* (= *A. oxycedri*)	Dowding (1931)	The account is very brief and without any figures to illustrate the development of the embryo sac
Dendrophthora gracile[1]	York (1913)	—
Korthalsella Dacrydii	Rutishauser (1935)	Stevenson (1934) has, however, figured a tetrad of four megaspores in *K. Lindsayi*[2]
Viscum album	Steindl (1935)	—
V. articulatum (= *Korthalsella opuntia*)	Steindl (1935)	—

[1] *D. opuntioides* (York, 1913) also shows the same type of development, but according to the author no reduction seems to occur here and the development is apomictic. For the same reason *Balanophora elongata* and *B. globosa* (Ernst, 1914) have not been included in this list.

[2] The Loranthaceae, Balanophoraceae and Santalaceae are among the most difficult angiosperms for embryological investigation, and the variations of development reported in them are to be attributed chiefly to: (1) environmental differences caused by their parasitic or semi-parasitic habit, (2) difficulties of technique (fixing as well as staining), and (3) inadequacy of material. Botanists from Europe and America have usually collected the material from the tropics and then studied it at home only to find that some of the most important stages are still missing. It seems certain that if the same plants were to be carefully reinvestigated by botanists resident in the country where they grow, many new facts would be discovered.

	HYDNORACEAE	
**Prosopanche bertoniensis*	Chodat (1916)	—
	NYCTAGINACEAE	
s *Oxybaphus nyctagineus* s *Mirabilis jalapa*	Rocèn (1927, p. 23)	Usually Normal-type; *Allium*-type and *Adoxa*-type both occur rarely *as abnormalities*
	CARYOPHYLLACEAE	
***Sabulina longifolia*	Fischer (1880)	But see Rocén (1927, p. 113), who considers this to belong to the Normal-type
	CRUCIFERAE	
s *Ionopsidium acaule*	Corti (1930)	Usually Normal-type; *Allium*-type occurs *only in exceptional cases*
	DROSERACEAE	
s *Dionaea muscipula*	Smith (1929)	Usually Normal-type; but Fig. 42 of the author indicates that *sometimes Allium*-type may also occur
	CRASSULACEAE	
Sedum fabaria *S. populifolium* *S. populifolium* var. *Notarjanni*	Mauritzon (1933, p. 27)	—
	SAXIFRAGACEAE	
***Ribes pallidum*	Himmelbaur (1911)	Mauritzon (1933) thinks it to be normal
	MIMOSACEAE	
***Acacia rostellifera*	Jönsson (1879–80)	This is almost certainly incorrect, for Guignard (1881) reported Normal-type in three species of *Acacia* and recently Newman (1934) has done the same in *A. baileyana*
	PAPILIONACEAE	
***Lupinus luteus* ***L. polyphyllus*	Guignard (1881)	This is very doubtful. Both species need to be reinvestigated
***Lathyrus odoratus*	Jönsson (1879–80)	This is certainly incorrect, for Roy (1933) finds Normal-type
	RUTACEAE	
s *Xanthoxylum alatum* s *X. Bungei*	Mauritzon (1935)	In both cases usually Normal-type occurs. *Allium*-type *only as an abnormality*
	MALPIGHIACEAE	
Galphimia gracilis	Stenar (1937)	—
	EUPHORBIACEAE	
Euphorbia mauritanica	Ventura (1934*a*)	—
	CELASTRACEAE	
***Evonymus latifolius*	Jönsson (1879–80)	As pointed out by Andersson (1931), this is incorrect
	BALSAMINACEAE	
**Impatiens sultani*	Ottley (1918)	Schürhoff (1931, p. 328) thinks this to be a mistake and regards the development as normal
	RHAMNACEAE	
Zizyphus sativa	Chiarugi (1930)	—

	DATISCACEAE	
Datisca cannabina	Himmelbaur (1909), Mauritzon (1936*a*)	—
	MYRTACEAE	
s *Myrtus communis*	Greco (1930)	*Only occasionally*; usual course of development is perfectly normal
	UMBELLIFERAE	
Bupleurum aureum	Håkansson (1923)	In *B. junceum* and most other plants of the family Normal-type occurs
	OLEACEAE	
Olea chrysophylla *O. europaea*	Andersson (1931, p. 56)	—
s *Syringa vulgaris*	Andersson (1931, p. 53)	*Only sometimes*
	ASCLEPIADACEAE	
**Cynanchum vincetoxicum*	Seefeldner (1912, p. 275)	Needs confirmation
	CONVOLVULACEAE	
**Cuscuta reflexa*	Johri & Nand (1934)	Fedortschuk (1931) and Smith (1934) report Normal-type in the species investigated by them
	POLEMONIACEAE	
**Polemonium coeruleum*	Jönsson (1879–80, p. 17)	An old account that needs confirmation
	BORAGINACEAE	
Anchusa officinalis	Svensson (1925)	—
Lycopsis arvensis	Svensson (1925)	—
	VERBENACEAE	
**Avicennia officinalis*	Karsten (1891)	Needs reinvestigation
**A. marina* var. *alba*	Junell (1934)	Needs confirmation
	SOLANACEAE	
Datura	Satina & Blakeslee (1935)	—
Nicotiana rustica	Persidsky & Modilewski (1934)	Sometimes more than eight nuclei are reported to occur
N. glauca	Modilewsky (1936)	—
	GESNERIACEAE	
s **Rhytidophyllum crenulatum*	Cook (1907)	The author saw only one instance of *Allium*-type and states that mostly the development is of the *Adoxa*-type
	RUBIACEAE	
**Scyphiphora hydrophyllacea*	Karsten (1891)	An old account that needs confirmation, specially since the more recently investigated plants of this family are normal
	CAPRIFOLIACEAE	
Viburnum acerifolium *V. lantana*	Suneson (1933)	

	COMPOSITAE	
Erigeron glabellus	Carano (1921)	*Peperomia*-type also occurs
E. alpinus	Chiarugi (1927*b*)	
E. Coulteri	Holmgren (1919)	
E. unalaschkensis	Holmgren (1919)	
Vittadinia triloba	Palm (1922)	Earlier stages in development show some interesting features which deserve further study
	POTAMOGETONACEAE	
**Potamogeton foliosus*	Wiegand (1900)	Seems incorrect; all other plants of this family are normal
	NAJADACEAE	
**Najas flexilis*	Campbell (1897)	Needs confirmation
	ALISMACEAE	
Alisma plantago	Dahlgren (1928), Johri (1936*b*)	All of these plants show a reduction in the chalazal part of the embryo sac, which is commonly 6-nucleate. Narasimha-Murthy (1933) reports only 8-nucleate embryo sacs in *L. obtusifolium*, but this is regarded as incorrect by Johri (1935 *a*)
A. plantago-aquatica	Johri (1936*b*)	
Damasonium alisma	Dahlgren (1928)	
Echinodorus macrophyllus	Dahlgren (1934)	
E. ranunculoides	Dahlgren (1928)	
Limnophyton obtusifolium	Johri (1935*a*)	
Sagittaria sagittifolia	Dahlgren (1934), Johri (1935*b*)	
S. guayanensis	Johri (1935*c*)	
S. latifolia	Johri (1935*c*)	
S. graminea	Johri (1936*b*)	
	BUTOMACEAE	
Butomopsis lanceolata	Johri (1936*a*)	—
Limnocharis emarginata (=*L. flava*)	Johri's (1936*a*) interpretation of the figures of Hall (1902)	—
Hydrocleis nymphoides	Johri (in the press)	—
	HYDROCHARITACEAE	
**Hydromystris stolonifera*	Tassi (1900)	Needs reinvestigation
	GRAMINEAE	
***Cornucopiae nocturnum*	Guignard (1882)	From Hérail's (1889) statement Normal-type seems more likely
***Melica nutans*	Fischer (1880)	The account is old and seems to be incorrect; all later investigated Gramineae have turned out to be normal
***M. altissima*		
	COMMELINACEAE	
***Commelina stricta*	Guignard (1882)	Maheshwari & Singh (1934) report Normal-type in *C. benghalensis* and it is almost certain that *C. stricta* will also be found to be so on reinvestigation
	PALMACEAE	
Nipa fruticans	Radermacher (1925)	—
Chamaedorea latifolia	Jönsson (1879–80)	—

	ARACEAE	
**Antherurus attenuatus*	Jönsson (1879–80)	—
**Arisaema triphyllum*	Gow (1908*a*)	Pickett (1915) has, however, figured a tetrad of megaspores
**Arum maculatum*	Jönsson (1879–80)	But see remarks by Schnarf (1931, p. 282)
**Dieffenbachia seguine*	Campbell (1900)	—
**Homalonema argentea*	Gow (1913)	—
H. alba, *H. rubra*	Jüssen (1928)	—
**Nephthytis Gravenreuthii*	Gow (1908*b*)	The author's statements are not quite clear
**Pistia stratioites*	Shadowsky (1931); *sometimes*	See p. 372 of this paper
	LEMNACEAE	
Lemna trisulca	Jönsson (1879–80)	—
Wolffia arrhiza	Gupta (1935)	—
	LILIACEAE	
Allium fistulosum	Strasburger (1879)	—
A. odorum	Schürhoff (1922), Modilewski (1925)	—
A. oleraceum	Stenar (1932)	—
A. roseum var. *bulbilliferum*, *A. nigrum*, *A. subhirsutum*, *A. neapolitanum*, *A. schoenoprasum*, *A. triquetrum*	Messeri (1931)	—
A. rotundum, etc.	Weber (1929)	The author has investigated several species, but all the stages were not seen by her
A. ursinum	Schniewind-Thies (1901)	—
***Convallaria majalis*	Wiegand (1900)	See, however, Schniewind-Thies (1901, p. 5)
s *Galtonia candicans*	Schniewind-Thies (1901); *sometimes*	Usually Normal-type
Nothoscordum fragrans	Messeri (1931), Stenar (1932)	—
Ornithogalum pyrenaicum	Guignard (1882)	—
Paris quadrifolia	Ernst (1902)	—
Ruscus aculeatus	De Philippis (1936)	—
Scilla campanulata, *S. hyacinthoides* var. *coerulea*	McKenney (1904)	—
S. nonscripta	Hoare (1934)	—
S. sibirica	Schniewind-Thies (1901)	—
S. nutans, *S. patula*	Guignard (1882)	—
S. hispanica	Treub & Mellink (1880)	—
Trillium grandiflorum	Ernst (1902)	—
T. cernuum	Heatley (1916)	—
T. recurvatum	Heatley (1916)	Coulter & Chamberlain (1903) report this as normal, but this is incorrect
T. sessile	Spangler (1925)	—
Tulbaghia violacea	Stenar (1933)	

	AMARYLLIDACEAE	
Crinum latifolium	Stenar (1925*b*), also Tomita (1931)	—
C. longifolium	Stenar (1925)	—
s *Hypoxis decumbens*	Stenar (1925)	—
Furcraea andina	Nevins (1927)	—
Narcissus micranthus	Guignard (1882)	—
N. Tazetta	Treub & Mellink (1880), Guignard (1882)	—
**Pancratium maritimum*	Shadowsky (1925*a*)	See p. 372 of this paper
	BURMANNIACEAE	
Burmannia candida	Ernst & Bernard (1912)	—
	ORCHIDACEAE	
Cypripedium guttatum	Prosina (1930)	—
C. calceolus	E. Oberhammer (unpubl.)	—
C. spectabile, *C. parviflorum*, *C. pubescens*, *C. candidum*	Pace (1907)	See remarks on p. 374 of this paper
Epidendrium variegatum	Sharp (1912)	—
s *Epipactis latifolia*	Vermoesen (1911); *only sometimes*	—
s *E. pubescens*	Brown & Sharp (1911); *only sometimes*	—
s *Gyrostachis cernua*, s *G. gracilis*	Pace (1914); *sometimes*	—
Neottia nidus avis	Modilewski (1918)	—
s *Orchis sambucina*	Afzelius (1916); *only rarely*	—
O. praetextum	Afzelius (1916)	—
Paphiopedilum insigne	Afzelius (1916)	—
P. Leeanum, *P. spicerianum*, *P. barbatum*, *P. villosum*, *P. venustum*	Francini (1931)	—

Podostemon-type

Among bisporic embryo sacs, *Podostemon subulatus*,[1] *Hydrobium* (=*Zeylandium*) *olivaceum*, *Farmeria metzgerioides* (Magnus, 1913) and *Weddelina squamulosa* (Chiarugi, 1933) show the greatest reduction. In these cases the nucleus of the lower dyad cell divides only twice, resulting in four nuclei that organize into the egg apparatus and a single polar nucleus. There is no nucleus at the chalazal end. We have a total of only three nuclear divisions between the megaspore mother cell and the egg and therefore these embryo sacs cannot be classified under the *Allium*-type. It is clear, however, that this is the result of only a continued tendency towards reduction and the family Podostemonaceae itself shows a reduced *Allium*-type in some plants and the *Podostemon*-type in others.

[1] Since this was written, Hammond (1937) has described the occurrence of a 5-nucleate embryo sac in *Podostemon ceratophyllum*, similar in origin to the type found by Went in the Podostomaceae investigated by him.

The problematical embryo sac of *Dicraea elongata* (Magnus, 1913) may also be considered here. All stages have not been found in this case, but according to the statements of Magnus the nucleus of the upper dyad cell divides to form one synergid and the egg, while the lower cell divides anticlinally to form two cells that may be called antipodals. A polar nucleus does not exist and consequently double fertilization is not possible. As pointed out by Schnarf (1936) the occurrence of such an embryo sac does not seem to be very probable and a thorough reinvestigation is necessary before these conclusions can be accepted.

Trisporic embryo sacs

Till recently there was no case known where three megaspore nuclei took part in the formation of an embryo sac. Mauritzon (1933, p. 29) has found that this happens in *Aeonium* (=*Aldasorea*) *guttatum* in a sufficient number of cases to justify its being classified under a new type.

This plant was found to be rather variable in its behaviour and three different types of development may occur:

(1) There is no wall formed after the heterotypic division. After the homotypic division walls are laid down in such a fashion that the middle cell is binucleate. It is, however, the chalazal megaspore that functions and in this instance the development corresponds to the Normal-type.

(2) Wall formation was not seen to occur at all after the reduction divisions. The further history of such cases was not followed, but it may lead to the formation of an 8-nucleate (*Adoxa*-type) or 16-nucleate (*Peperomia*-type) embryo sac.

(3) In still other cases only one wall is laid down after the reduction divisions and that in such a way as to form a 3-nucleate cell at the top and one uni-nucleate cell at the bottom. Usually the chalazal cell develops further and gives rise to an 8-nucleate embryo sac of the normal type.

In a few cases, however (and it is these with which we are concerned here), it is the micropylar cell that functions and each of the three nuclei divides twice so as to form a total of twelve nuclei, which give rise to a normal egg apparatus of three cells, a group of three antipodal cells and *six* polar nuclei.

Mauritzon thinks that this mode of development must be referred to a new type by itself.

Peperomia-type[1]

Campbell (1899) was the first to call attention to certain peculiarities in the embryo sac of *Peperomia pellucida*, but Johnson

[1] See Fig. 6 for a diagrammatic representation of the variations under this type.

(1900*b*) gave a fuller account of the development. The four nuclei formed after the first two divisions of the megaspore mother cell are arranged like the spores of a tetrad and are connected in the beginning by strands of granular cytoplasm. These divide to form eight nuclei imbedded in the peripheral cytoplasm, each of which now divides again resulting in sixteen free nuclei, of which one forms the egg, another gives rise to a synergid, eight fuse to form a secondary nucleus, and the remaining five are cut off as antipodal cells.

A few years later the same author (Johnson, 1914) described the embryo sac of *P. hispidula* and found that the egg and synergid are formed as in *P. pellucida* but the remaining fourteen nuclei all fuse together in the centre to form a larger secondary nucleus.

Four other investigators, viz. Brown (1908), Fisher (1914), Häuser (1916) and Abele (1923, 1924), have examined several other species of this genus with results essentially similar to those in one or the other of the above two cases. Evanescent cell plates, often formed between the nuclei in the first two divisions, indicate that they should be regarded as equivalent to the nuclei of the once-walled spores. It is to be noted that in spite of the larger number of nuclei in the sac, the egg is removed from the megaspore mother cell by only four divisions as against the normal five.

The following table summarizes our knowledge of the nuclear arrangement and embryo sac organization in the species of *Peperomia*

Name of species	Author	Egg	Synergids	Polar nuclei	Antipodal nuclei
P. arifolia	Brown (1908)	1	1	8	6
P. blanda	Fisher (1914)	1	1	±6	±8
**P. Fraseri* var. *residiflora*	Fisher (1914)	1	1	8–6	6–8
**P. galioides*	Fisher (1914)	1	1	8–6	6–8
P. hispidula	Johnson (1914)	1	1	14	Nil
P. incana	Abele (1924)	1	1	?14?[1]	
P. Magnoliifolia	Häuser (1916)	1	1	8	6
P. metallica	Abele (1923)	1	1	6	8
P. Ottoniana	Brown (1908)	1	1	8	6
P. pellucida	Johnson (1900*b*)	1	1	8	6
P. reflexa	Fisher (1914)	1	1	8–6	6–8
**P. residiflora*	Häuser (1916)	1	1	8	6
P. scandens	Fisher (1914)	1	1	8–5	6–9
P. sintensii	Brown (1908)	1	1	8	6
P. verschaffeltii	Abele (1923)	1	1	?14?	
P. verticillata	Fisher (1914)	1	1	8–6	6–8

* Denotes species that have not been very fully investigated.

[1] Authors (Fisher, 1914; Abele, 1924) have called attention to the difficulty in determining the exact number of nuclei that fuse to form the secondary nucleus and others that remain as antipodals. This is due to the fact that the nuclei do not always fuse at one and the same time and the number of lobes on the secondary nucleus or the nucleoli within it are not sufficiently accurate indicators of the number of nuclei that entered into the fusion.

so far studied. It is rather remarkable that in all cases only a single synergid is present. Some authors have expressed the view that the egg and synergid are sister cells in *Peperomia*, but this has been taken for granted merely because of their relative position and this point still remains to be proved one way or the other.

At present tetrasporic embryo sacs with sixteen nuclei are known in seven other families besides the Piperaceae: Euphorbiaceae,

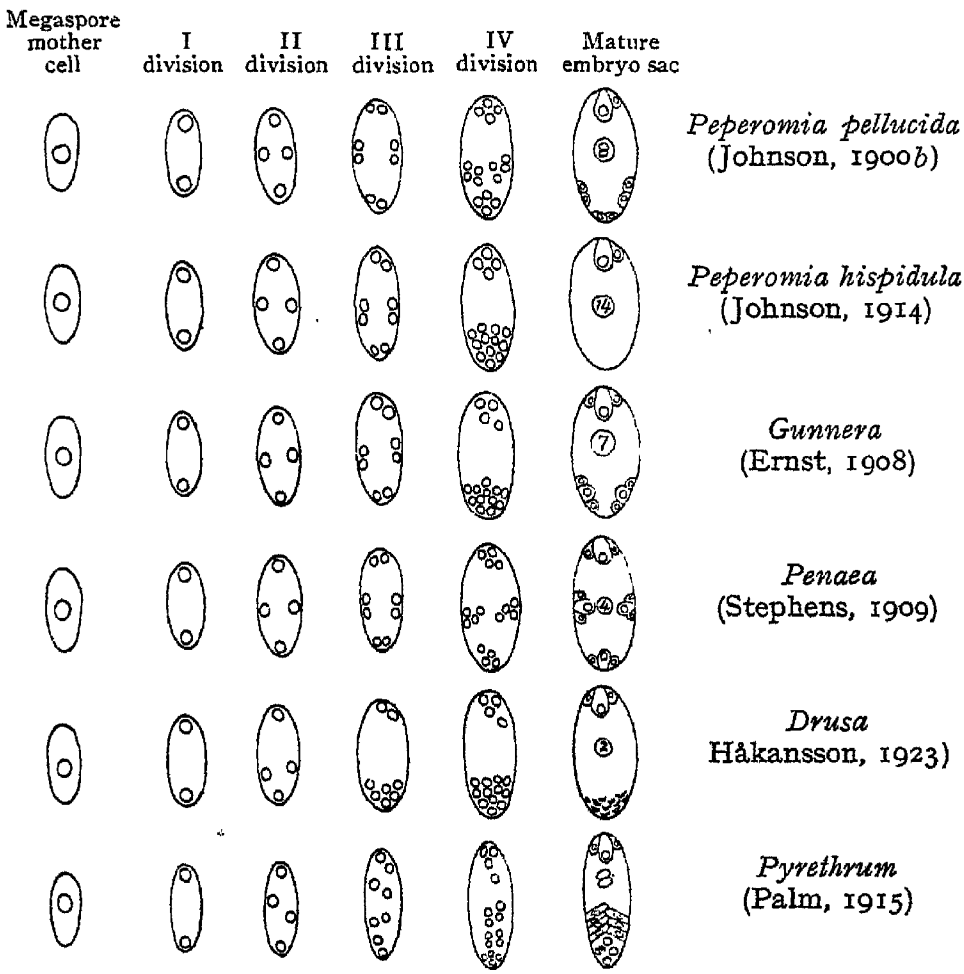


Fig. 6. Diagrams to show more important types of variations found under the *Peperomia*-type of embryo sac.

Penaeaceae, Gunneraceae, Malpighiaceae, Umbelliferae, Compositae and Liliaceae. We shall group the abnormalities in such a way as to call attention to their similarities.

Penaea-form. Miss Stephens (1909) found that in three genera of the Penaeaceae (*Penaea ovata*, *P. mucronata*, *Brachysiphon imbricatus*, *Sarcocolla squamosa*, *S. formosa* and *S. furcata*), the first two divisions form a tetrad of nuclei just as in *Peperomia*. These separate and each divides twice to form a group of four nuclei. Cell walls are now organized around three nuclei of each quartet;

the remaining four move to the centre and fuse to form a secondary nucleus.

This type of development has since been reported in some other plants also, viz. *Acalypha* sp. (Arnoldi, 1912); *A. australis* (Tateishi, 1927); *A. indica* (Maheshwari & Johri, 1937; see also Maheshwari, 1935); *Euphorbia palustris* (Modilewski, 1911); *E. procera* (Modilewski, 1909*a*, 1910; Schürhoff, 1924); *E. virgata* (Modilewski, 1911); *Malpighia coccifera, M. urens,* and *Brunchosia nitida* (Schürhoff, 1924); *Azorella trifurcata* (Håkansson, 1927).

Tateishi (1927) and some others have expressed the opinion that such an embryo sac corresponds to four monosporic tetranucleate embryo sacs arranged at right angles to one another. The important point to determine is whether the "egg" in the other three triads besides the one at the micropylar end can function and give rise to an embryo. The figures of most of the authors cited above do not always show an appreciable differentiation into egg and synergids in these triads.

Gunnera-form. The embryo sac of *Gunnera* was first studied by Schnegg (1902), but the full details were available only after the work of Modilewski (1908) on *G. chilensis* and Ernst (1908) and Samuels (1912) on *G. macrophylla*. Of the sixteen nuclei formed after the fourth division, three at the micropylar end form a normal egg apparatus, the fourth descends and fuses with six of the chalazal nuclei to form a large secondary nucleus and the remaining six are cut off to form antipodal cells. Ernst thinks that they may be said to represent two egg apparatuses, but this would hardly be acceptable till we know more about their function.

Drusa-form. The embryo sacs of *Drusa oppositifolia* (Håkansson, 1923), *Bowlesia tenera* (Håkansson, 1927), *Majanthemum bifolium* (Stenar, 1934) and *Mollatus japonicus* (Ventura, 1934*b*) are somewhat different. After the reduction divisions are over, three of the megaspore nuclei pass down to the chalazal end and only one remains at the micropylar. At the 8-nucleate stage we have, therefore, a 2+6 arrangement and after the last division four nuclei are seen at the micropylar end and twelve at the chalazal. The former group gives rise to the egg apparatus and leaves one nucleus free to move down and fuse with another from the lower end; the remaining eleven nuclei are cut off as antipodals.

Stenar (1934) has seen that in *Smilacina stellata* also the development proceeds in an exactly similar fashion up to the 8-nucleate stage and shows a 2+6 arrangement of the nuclei. Further development could not be traced but he thinks it highly probable that here

also there is one more division resulting in a 16-nucleate embryo sac of the same kind as in *Majanthemum bifolium.*

Pyrethrum-form. Palm (1915) gave a full account of the development in *Pyrethrum parthenifolium* var. *aureum* (=*Chrysanthemum parthenium* var. *aureum*). After the reduction divisions are over, the four nuclei become arranged in a single row and each of them divides to form a group of four. The mature embryo sac contains a normal egg apparatus, two polar nuclei, seven uninucleate antipodal cells and one large antipodal cell with four nuclei. The embryo sacs of *Erigeron dubius* (Tahara, 1921), *E. alpinus* (Chiarugi, 1927*b*), *E. eriocephalus, E. politus* (Holmgren, 1919) seem to be similar, although they have not been so thoroughly studied.

Embryo sacs of the *Peperomia*-type, with a greater or smaller number of nuclei than the usual sixteen, may occur occasionally as abnormalities, but *Tanacetum vulgare* (=*Chrysanthemum vulgare*) studied by Palm (1915) seems to be the only case where 12- and 14-nucleate embryo sacs are of common occurrence. The development differs from *Pyrethrum* in the fact that one or both of the megaspore nuclei at the chalazal end of the embryo sac fail to undergo one division. *Erigeron karwinskianus* (Carano, 1921) shows the opposite condition, for here the number may go up to twenty, which is due to a fifth division of some of the chalazal nuclei.

Fritillaria-type

Until recently it used to be thought that in *Lilium* the development of the embryo sac is of a very simple type. Treub & Mellink (1880) reported that in *L. bulbiferum* the megaspore mother cell does not form the usual tetrad of megaspores, but undergoes only three divisions (against the usual five) to give rise to an octo-nucleate embryo sac. After Treub & Mellink's work, this method of development was reported in many other plants, and for the sake of convenience it began to be known as the "*Lilium*-type".

A thorough investigation of the embryo sac of *Fritillaria persica* and *Lilium candidum* (Bambacioni, 1928 *a, b*) showed that instead of the egg being removed from the megaspore mother cell by three divisions, four actually intervene. After the hetero- and homotypic divisions, the four megaspore nuclei arrange themselves in such a fashion that only one remains at the micropylar end while the other three migrate to the chalazal end of the sac. All of these now divide simultaneously, but the spindles of the three chalazal nuclei fuse to form a large common spindle, which is multipolar in the beginning but soon becomes bipolar. As a result of this division, the embryo

sac is *again 4-nucleate*, the two micropylar nuclei being haploid and also smaller than the chalazal nuclei which are triploid. One further division occurs and the eight nuclei now formed are arranged in two groups: a micropylar quartet of four haploid ones and a chalazal quartet of four triploid ones. All the cells of the egg apparatus and the upper polar nucleus are consequently haploid; the lower polar nucleus and the antipodals are triploid, but two of the latter, formed by the division of the lowest chalazal nucleus of the secondary 4-nucleate stage, are usually very ephemeral from the very beginning of their formation.

A couple of years later, Bambacioni & Giombini (1930) described the same phenomenon in *Tulipa gesneriana* and Bambacioni (1931) herself added *T. praecox* and *Lilium bulbiferum* to the list. Recently Cooper (1935) has investigated a number of species of *Lilium* and finds the same type of development in all of them. It is interesting to note that Guignard (1891), Sargant (1896), Coulter (1897) and Mottier (1898) saw abnormal appearances pointing towards the same conclusion in the species of *Lilium* investigated by them, but they thought these to be caused by some physiological or pathological conditions and failed to pursue the point further.

In a still more recent paper, Romanov (1936) reports the same type of development in *Gagea ova* and *G. graminifolia* and considers its occurrence to be very probable in *G. tenera* also, which was however not so fully studied. By comparing Stenar's (1927) figures of *G. lutea* with his own preparations of these three species, he comes to the conclusion that the same type occurs in Stenar's plant also.[1]

As Dr Cooper (1935) has remarked, there is now very strong reason to think that this type of development is characteristic of the genus *Lilium* as a whole, and it has been suggested (Maheshwari, 1936, and others) that the name "*Lilium*-type" be discarded altogether. What we so far understood by this term should now be called the "*Adoxa*-type", since *A. moschatellina* (Jönsson, 1879–80) was the first plant in which all the four megaspore nuclei were observed to divide once to give rise to an 8-nucleate embryo sac.

The new cases, described above, have tetrasporic embryo sacs, in which four divisions intervene between the megaspore mother cell and the egg. The result is, however, not a 16-nucleate embryo sac as in *Peperomia*, but a normal-looking one whose 8-nucleate condition is brought about by special conditions, i.e. the interposition

[1] Since this was written, Westergård (1936) has added *Gagea minima* also to the list and Oikawa (1937) has demonstrated the occurrence of the *Fritillaria*-type in *Cardiocrinum cordatum*.

of a secondary 4-nucleate stage. We think that this type is distinctive enough to earn a new name—the "*Fritillaria*-type".

Although typically the number of nuclei is eight, cases of a reduction in their number are not infrequent (see Fig. 7). Of the two chalazal nuclei of the secondary 4-nucleate stage, the innermost is usually in a degenerating condition right from the time of its formation. In *Fritillaria*, it succeeds in going through a normal division, but in *Lilium* (Cooper, 1935) the division is more or less abortive. In *Gagea*

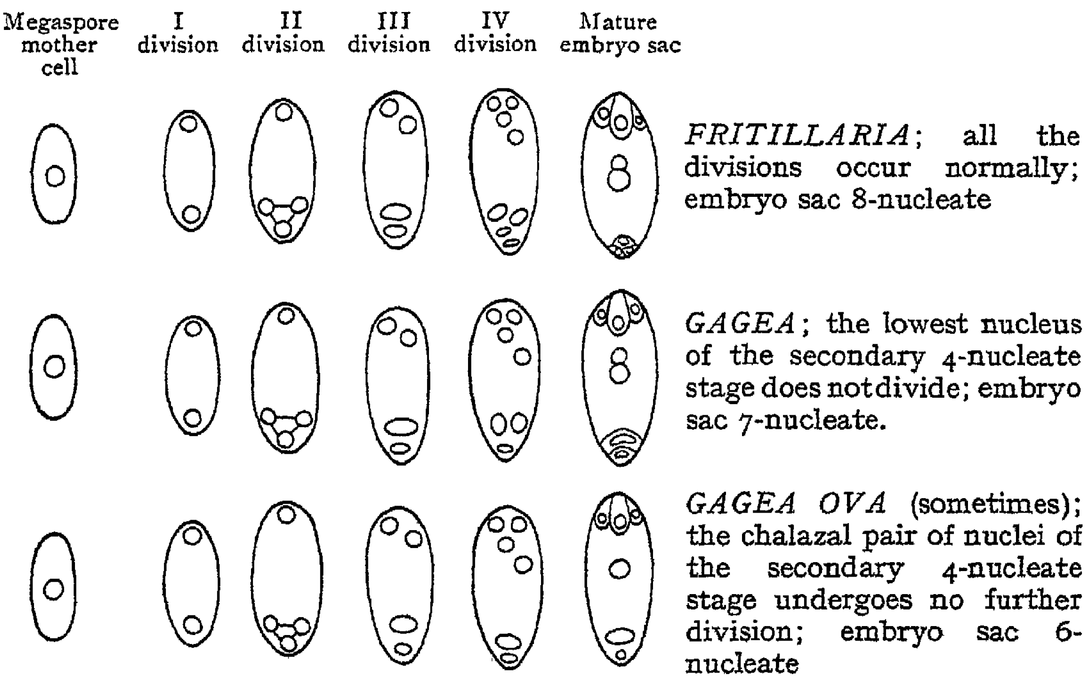


Fig. 7. Diagrams to show reduction of number of nuclei in chalazal part of embryo sac of *Fritillaria*-type.

this division commonly does not take place at all and the embryo sacs are 7-nucleate; finally there are some cases in *G. ova* where both of the chalazal nuclei fail to divide and the mature embryo sac is therefore only 6-nucleate. All three conditions may also occur in the same plant, viz. *Myricaria germanica* (Frisendahl, 1912).[1]

The following appearances are thus quite characteristic of the *Fritillaria*-type:

(1) A 1+3 arrangement of the megaspore nuclei.

(2) A fusion of three spindles in the chalazal region resulting in a larger number of chromosomes on the equatorial plate.

(3) A secondary 4-nucleate stage, easily recognized by the greater size of the chalazal nuclei which are triploid.

[1] A further reduction may occur if the 3 chalazal nuclei of the primary 4-nucleate stage fuse to form a triploid nucleus, which does not undergo any further division. Such 5-nucleate embryo-sacs have been seen by Mauritzon (1936 *c*) in *Caulophyllum robustum*.

Now we may call attention to certain doubtful cases, which have been reported to belong to the *Adoxa*-type, but (judging from the figures and descriptions of the authors themselves) may really come under the *Fritillaria*-type.

Piper subpeltatum. Some of Palm's (1915) figures of this plant show a clear 1+3 arrangement of the megaspore nuclei and also an appreciable difference in the relative sizes of the micropylar and chalazal nuclei of the 4-nucleate stage. Schnarf (1931, p. 11) stated that the development here is not of the *Adoxa*-type as described by Palm, but of the *Fritillaria*-type. Others (Maheshwari, 1936; Romanov, 1936) have expressed an agreement with this view.

Piper betel var. *monoicum.* A glance at Johnson's (1910) Figs. 56, 7 and 58 (reproduced here as Fig. 8 *c*, *a*, *b*) shows that they were drawn in an incorrect sequence. Prof. Johnson (1910, p. 725) writes: "The four nuclei resulting from this second division of the embryo sac may sometimes remain near the ends of the sac, where they are formed, or there may be a single nucleus at one end and three at the other (Figs. 56, 58). Often, however, perhaps in half the cases seen, these nuclei may be closely grouped near the middle of the embryo sac (Fig. 57)." What remained a puzzle to Prof. Johnson 25 years ago, may now be explained as follows without even taking the trouble of reinvestigating the plant:

Fig. 57 (here Fig. 8*a*). Primary 4-nucleate stage; megaspore nuclei as seen just after the homotypic division is over.

Fig. 58 (here Fig. 8*b*). Later stage, megaspore nuclei showing 1+3 arrangement.

Fig. 56 (here Fig. 8*c*). End of mitosis leading to the formation of the secondary 4-nucleate stage; the chalazal nuclei are distinctly larger in size, which is obviously due to their triploid origin.

Piper medium. Johnson's (1902) Figs. 7 and 8 have again been drawn in an inverted sequence and Fig. 8 called by the author as a later stage, is actually the earlier one, as is also borne out by the size of the nucellus and the embryo sac itself. Fig. 7 shows the telophase of the mitosis leading to the formation of the secondary 4-nucleate stage.

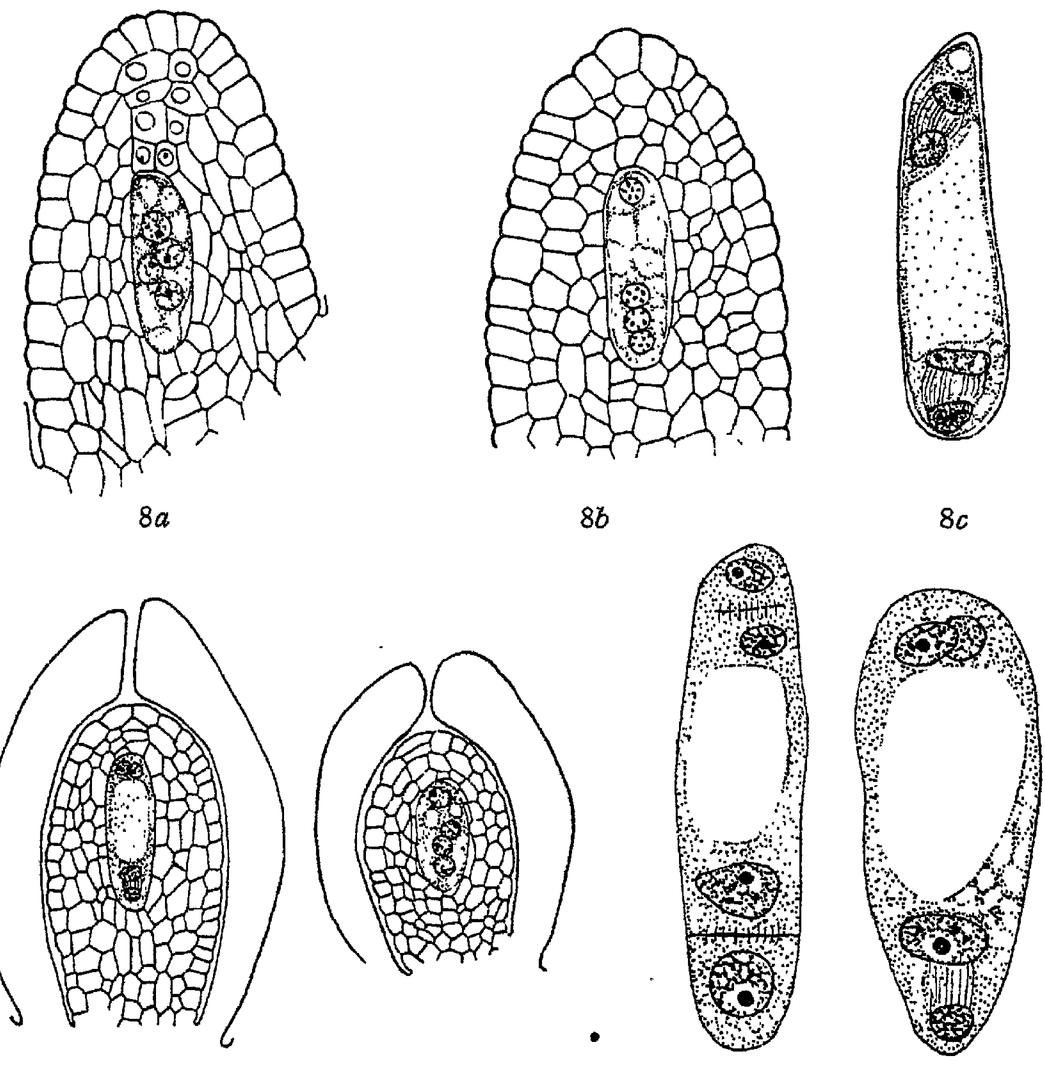
Heckeria umbellata. In this case also Johnson (1902) made a similar mistake. His Fig. 21 (reproduced here as Fig. 9*b*) shows the megaspore nuclei passing into the 1+3 arrangement, while Fig. 20 (reproduced here as Fig. 9*a*) must be regarded as representing the telophase of the third division leading to the secondary 4-nucleate stage.

In all the three cases cited above Prof. Johnson must be given the credit of having accurately drawn what he saw, although he failed to give the correct interpretation due to the lack of certain important stages. It seems probable that the other species of *Heckeria* and *Piper* will yield similar results on a reinvestigation.

Euphorbia dulcis. Since Carano (1925, 1926) had actually seen

a fusion of the three spindles in the chalazal end of the sac, there seems to be no doubt that the *Fritillaria*-type occurs here (see also Schnarf, 1931, p. 11).

Myricaria germanica. Frisendahl's (1912) figures show the 1+3 arrangement as well as a difference in the relative size of the micropylar and chalazal pairs of nuclei of the 4-nucleate stage. There is,



8*a* 8*b* 8*c* 9*a* 9*b* 10*a* 10*b*

Figs. 8–10. Fig. 8 *a, b, c*. Some stages in development of embryo sac of *Piper betel* var. *monoicum* (after Johnson, 1910). Fig. 9 *a, b*. *Heckeria umbellata* (after Johnson, 1902). Fig. 10 *a, b*. *Medeola virginica* (after MacAllister, 1914).

therefore, no doubt that the *Fritillaria*-type occurs here, but this plant seems to be rather variable in its behaviour and therefore a reinvestigation is likely to give interesting results.

Tamarix. Mauritzon (1936*a*), who investigated *T. tetrandra* and five other species of the same genus, reports the *Adoxa*-type in every case. Joshi & Kajale (1936) have, however, been able to demonstrate that in *Tamarix dioica* the development is of the *Fritillaria*-type.

Since Mauritzon's figures do not give any indication of a 1+3 arrangement nor a difference in size of the nuclei at the two ends of the embryo sac, it is not possible to go further into the question and a fuller investigation of the genus *Tamarix* seems to be desirable.[1]

Rudbeckia hirta. In a preliminary note entitled "Ein neuer Embryosacktypus (bei *Rudbeckia hirta* L.)", Palm (1934) has recently reported a type of development in this Composite, which does not come under any of the existing types. No walls are laid down during the reduction divisions (Fig. 11 *a–d*). The micropylar nucleus remains in its original position, but the two lateral nuclei move down to the

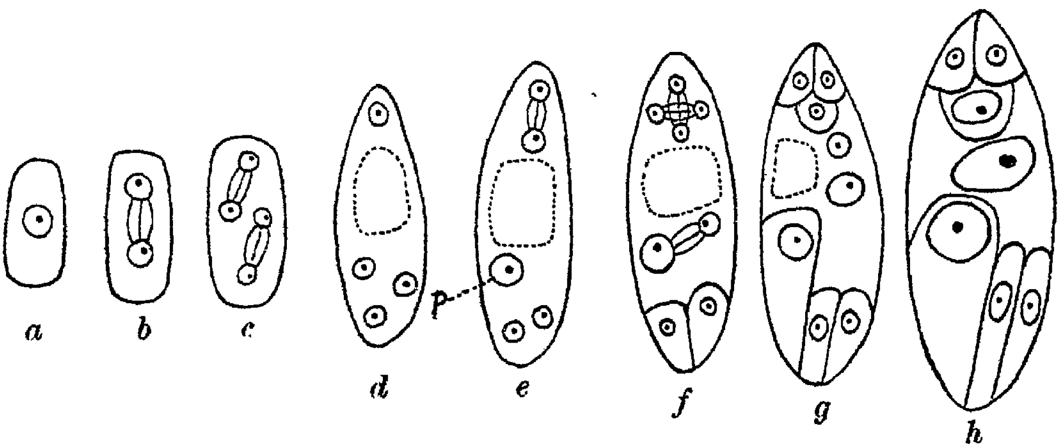


Fig. 11 *a–h*. Development of embryo sac of *Rudbeckia hirta* (after Palm, 1934).

bottom of the embryo sac giving the characteristic 1+3 arrangement (Fig. 11 *d*). Two of these nuclei, according to Palm, become cut off by membranes to give rise directly to antipodal cells, while the third (*p*) enlarges and takes up a more central position. The micropylar megaspore nucleus quickly undergoes two divisions to form the egg apparatus and the upper polar nucleus and at the time of its second division, "*p*" divides to give rise to the lower polar nucleus and one large antipodal cell (Fig. 11 *e–h*).

Palm's fuller paper will doubtless be awaited with interest. Meanwhile it may be said that this embryo sac shows some significant similarities to that of *Fritillaria* and *Lilium*, i.e. (1) a 1+3 arrangement of the megaspore nuclei, (2) lower polar nucleus much larger than the upper, and (3) one antipodal cell larger than the other two.

Clintonia borealis. Several embryologists (Schnarf, 1931, 1936; Stenar, 1934) have expressed doubts about the reliability of Smith's (1911) observations on this plant. According to the original account, three of the megaspore nuclei formed after reduction degenerate, while the fourth, situated at the micropylar end, undergoes two divisions to form four nuclei, which give rise to the egg apparatus and a single polar nucleus.

[1] My pupil, Prof. V. Puri of Meerut, has made a detailed study of *Tamarix chinensis* and finds his observations to be in accordance with those of Joshi & Kajale. His full paper is now in the press.

This satisfies the requirements of the *Oenothera*-type, and if Smith's observations are correct *Clintonia* should be regarded as an interesting example of a monosporic tetranucleate embryo sac. Some of Smith's own figures are, however, capable of being interpreted differently. Thus:

Fig. 12*a*. Megaspore mother cell.

Fig. 12*b*. 1+3 arrangement of megaspore nuclei.

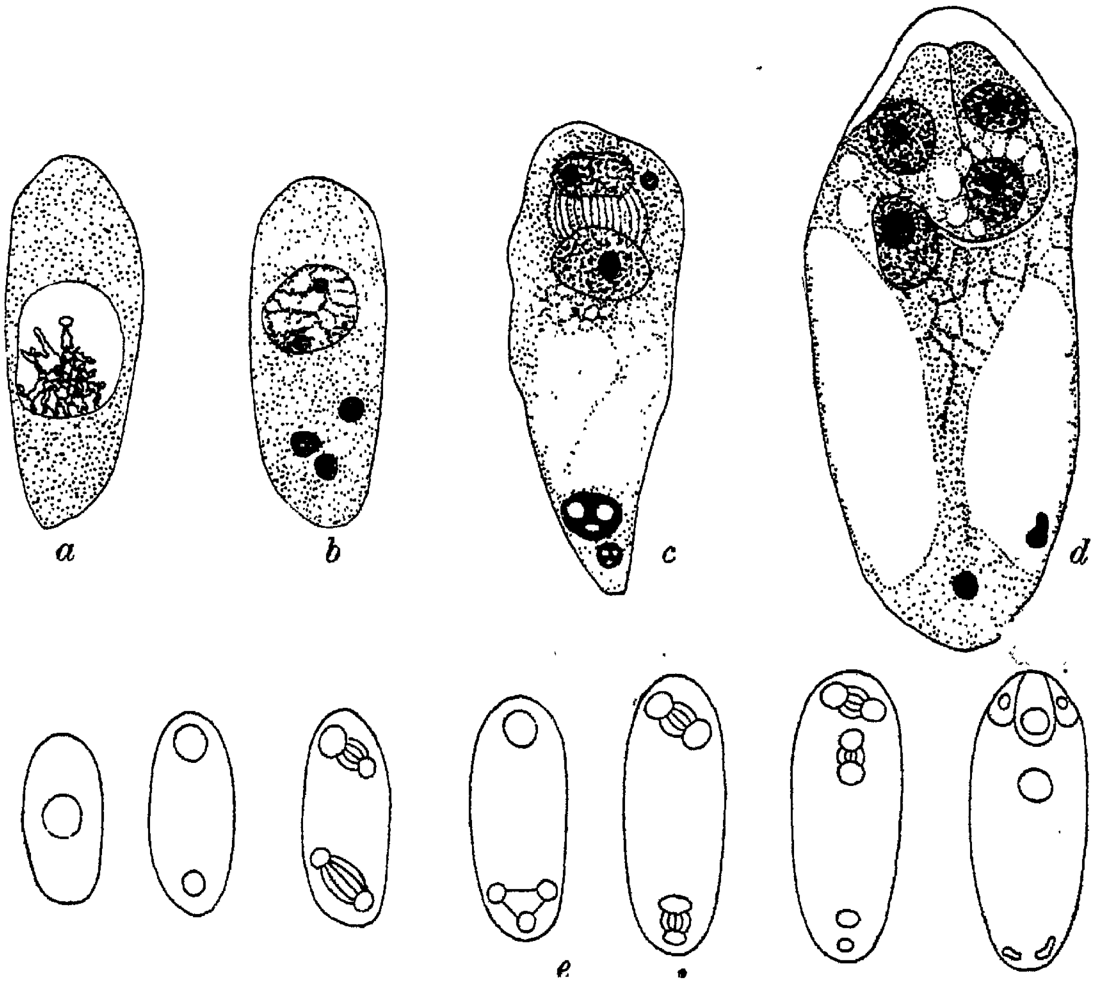


Fig. 12 *a–e*. *a–d*, some stages in development of embryo sac of *Clintonia borealis* (after Smith, 1911); *e*, series of diagrams from megaspore mother cell to mature embryo sac stage drawn to illustrate the interpretation of Smith's figures proposed in the text.

Fig. 12*c*. Perhaps a secondary 4-nucleate stage. The chalazal nuclei are not so large here as in *Lilium* and *Fritillaria* but this may be regarded as a natural consequence of the degenerating and feeble character of the three megaspore nuclei which have contributed to their formation. The three clear spaces in each nucleus may perhaps be taken to provide a strong evidence of their triploid nature.

Fig. 12*d*. Here we see the last remains of the chalazal pair of nuclei.

If the view put forth above is correct, we have in *Clintonia* an instance of a reduced embryo sac of the *Fritillaria*-type with six

nuclei instead of the normal eight (see Fig. 12*e* for an explanation of the interpretation proposed here). Such embryo sacs are known to occur in *Gagea* (Romanov, 1936) and *Myricaria* (Frisendahl, 1912) and their occurrence in *Clintonia* should be specially common in view of the degenerating nature of the megaspore nuclei from the very first.

Erythronium dens canis. Hrubý (1934), who investigated some stages in the development of the embryo sac, seems to have been ignorant of Bambacioni's work. The four reduction nuclei are arranged in the same manner as in *Fritillaria* (1+3). The mature embryo sacs are 8-nucleate. Intervening stages were not seen, but the author gives an ingenious explanation of the origin of the 8-nucleate stage by supposing that the micropylar nucleus divided twice to form a quartet, while at the chalazal end only one nucleus divided so that the number does not exceed four at this end also![1] Romanov (1936) thinks that the development, as far as it has been traced by Hrubý, indicates a close similarity with *Fritillaria* and we are in complete agreement with this view.

Fritillaria imperialis. Heinricher (1928) gave a figure of the primary 4-nucleate stage showing the 1+3 arrangement. Lenoir (1934) has demonstrated by chromosome counts that the development is similar to that in *F. persica*.

Fritillaria pudica. Sax's (1916) figures of triple fusion show that the lower polar nucleus is much larger than the upper.

Medeola virginica. McAllister (1914) reported *Adoxa*-type, but two of his figures (reproduced here as Fig. 10 *a*, *b*) seem to indicate that the *Fritillaria*-type occurs here also. A reinvestigation is of course necessary to clear up the point.

Adoxa-type

This type of development is characterized by all four megaspore nuclei undergoing one more division to form an 8-nucleate embryo sac with a normal mature organization. It was described almost simultaneously by Jönsson (1879–80) in *Adoxa moschatellina* and Treub & Mellink (1880) in *Lilium bulbiferum* and *Tulipa gesneriana*. Recent work, already outlined in the preceding pages, leaves no doubt that its occurrence in *Lilium* and *Tulipa* was mistaken, but the findings of Jönsson were confirmed by Lagerberg (1909). The old name "*Lilium*-type" has, therefore, to be replaced by "*Adoxa*-type".

Several other plants, in which older authors reported an *Adoxa*-type of development, have either turned out to be perfectly normal or found to pass through a secondary 4-nucleate stage (*Fritillaria*-type) before attaining the 8-nucleate condition. A few instances cited here will show the necessity for great caution in this respect.

[1] Note the similarity with Palm's interpretation of *Rudbeckia hirta*.

Schaffner (1897 *b*) in his work on *Typha latifolia* writes: "In the rear of the primary sporogenous cell, or the macrospore mother cell, a long axial row of cells is developed (Figs. 38–41). Often, if the section is not quite longitudinal, so that only three or four of the cells of the axial row are left back of the macrospore mother cell, there is an appearance as though there were a row of four or five megaspores. It is evident that extreme care must be taken not to mistake the large cells of the axial row for potential macrospores. It is possible that misinterpretations may sometimes have been made in this way. In *Typha* I was only able to determine conclusively the real fate of the macrospore mother cell by tracing out its development step by step, so closely did the cells of the axial row agree in size, structure, and staining reaction with the macrospore mother cells. The macrospore mother cell develops directly into the fertile macrospore without any division...." Dahlgren (1918) showed 20 years later that in spite of the "extreme care", which Schaffner claims to have exercised in formulating his conclusions, he made a mistake; *a tetrad of megaspore is formed* in *Typha* and the development is perfectly normal. Sporogenous cells may occasionally be arranged in a linear row (see Johri, 1935 *d*, on *Berberis nepalensis*) and get mistaken for megaspores, as Joshi & Rao (1934) did in the case of *Digera arvensis*.[1] However, if the nucleus can be observed in synizesis or some other stage of reduction division, the recognition of the megaspore mother cell stage is not difficult.

In recent years Heilborn (1921, 1928) has reported that in *Carica papaya* and some other species of this genus no tetrad of megaspores is formed but that all the four nuclei lie free and only one at the micropylar end divides again to form a 5-nucleate embryo sac! Agharkar & Banerji (1930) have nevertheless shown that tetrad formation does occur and Kratzer (1918) did the same.

An interesting situation has developed around the embryo sac of *Stellaria media*. Miss Gibbs (1907) reported that the development was of *Adoxa*-type, while Rocén (1927) who investigated this plant and many other members of the family Caryophyllaceae contended

[1] The need for accurate and detailed figures is particularly imperative in embryological work. Numerous instances can be cited in which the incorrect interpretations of certain authors have been corrected later by other investigators only if the figures were sufficiently accurate. The case of *Digera* itself has been fully dealt with by Puri & Singh (1935). It may be emphasized that the vast majority of angiosperms have a monosporic 8-nucleate embryo sac and deviations from this will be found only rarely if the material is well-fixed and the technique is adequate. When a different type of development *does* occur, statements must be fully supported by drawings of all important stages.

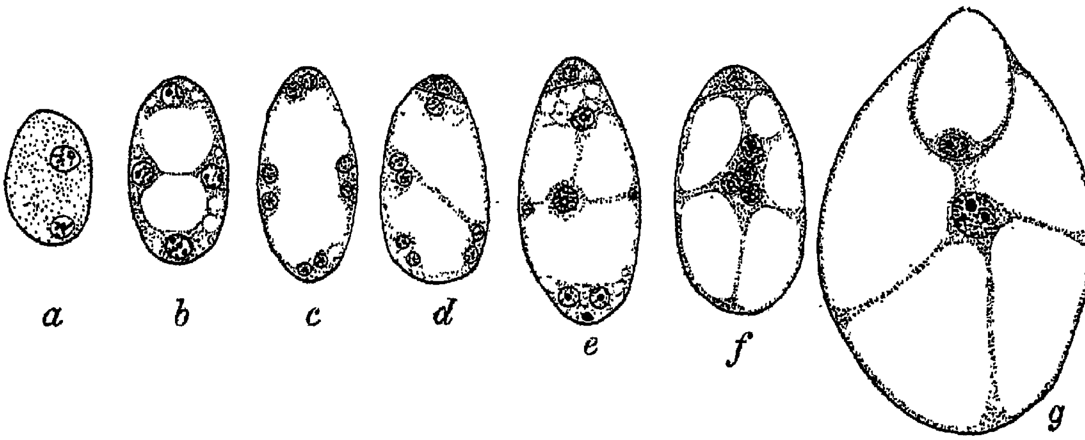
that it was perfectly normal. In a more recent work P. C. Joshi (1936) writes: "The further development of the megaspore mother cell is variable, and the results of the present investigation are at variance with those of Gibbs and Rocén. A row of three or four megaspores, formed as a result of the usual two successive divisions of the mother cell, was never seen. Only in a few cases the mother cell had divided to form two superposed daughter cells with a transverse or oblique wall in between (Figs. 35, 36)." One would conclude from this that according to Joshi the development is usually of the *Adoxa*-type and sometimes of the *Allium*-type. He does not however appear to be quite sure about it and states later that this "does not preclude the possibility that the two megaspores[1] may give rise to three or four megaspores". The whole thing needs careful reinvestigation. It would not be surprising if in a plant like *Stellaria media*, which is so variable in other respects, some differences may occur in the mode of embryo sac development also, but Joshi's figures do not seem to prove that it is so. On the other hand, the row of three cells shown in Fig. 35 and the four cells arranged in the form of a **T** in Fig. 36 would perhaps seem to speak for a normal type of development.

Among the plants belonging to the *Adoxa*-type, *Plumbago capensis* is of special interest. Dahlgren (1916), who was the first to investigate it, reported that the four megaspore nuclei formed after the reduction divisions directly entered into the organization of the mature embryo sac (*Plumbagella*-type). Haupt (1934), who has made a thorough study of this plant, finds that there is one more division, but of the eight nuclei so formed three degenerate and four fuse in the centre to form the secondary nucleus; the single nucleus remaining at the micropylar end is cut off to form the egg (Fig. 13*a–f*). The mature embryo sac is therefore only 2-nucleate (Fig. 13*g*), and without a complete series of stages such as Haupt found it would be easy to be led away to other interpretations of their origin.

The genus *Thesium* provides a good illustration of a reduction of the number of nuclei forming the mature embryo sac. Modilewski (1928, p. 67) called attention to the difficulty in finding the antipodals in *T. intermedium* and concluded that they were extremely ephemeral. In some cases he saw only two nuclei at the chalazal end, of which one functioned as the lower polar nucleus and the other disorganized. Schulle (1933) has confirmed these observations on *T. montanum*

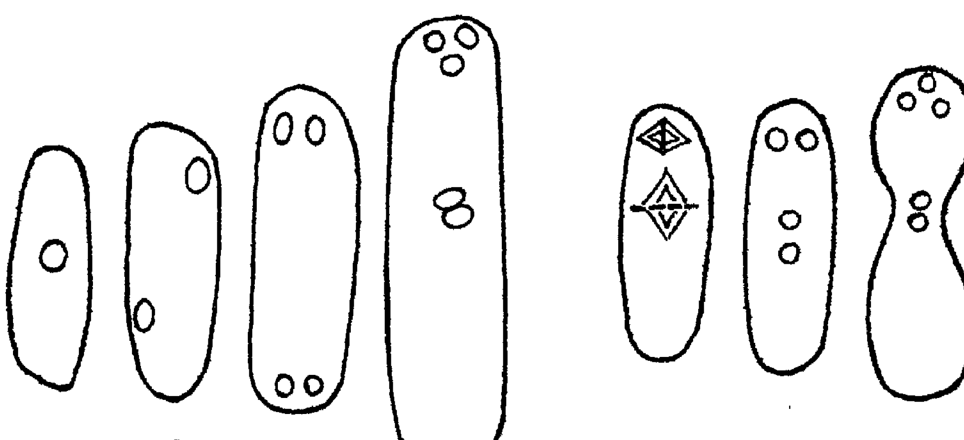
[1] As explained before, the two cells formed after the first division of the megaspore mother cell must be called dyad cells and not megaspores.

and finds that the 7- and 6-nucleate embryo sacs are due to a failure of division of one or both of the chalazal nuclei of the 4-nucleate stage. The conditions existing here offer a parallel to those in *Limnophyton obtusifolium* (Johri, 1935*a*), which has already been discussed under the *Allium*-type.



a *b* *c* *d* *e* *f* *g*

Fig. 13 *a–g*. Stages in development of embryo sac of *Plumbago capensis* (after Haupt, 1934).



a *b* *c* *d* *e* *f* *g*

Fig. 14 *a–g*. *a–d*, stages in development of embryo sac of *Eugenia jambos*; *e–g*, *E. bancana* (after Pijl, 1934).

The observations of Pijl (1934) recording a 5-nucleate embryo sac in *Eugenia jambos* and *E. bancana* cannot be regarded as conclusive. The author states that after the four megaspore nuclei have been formed, only one at the micropylar end divides again to give rise to the egg and one synergid, the second micropylar nucleus functions directly as a synergid and the two chalazal nuclei as polars. Pijl's figures, reproduced here on a slightly smaller scale (Fig. 14), are too diagrammatic and entirely inadequate to substantiate this conclusion. The fact that some other species of *Eugenia* investigated by the same author have a normal tetrad of megaspores, leads to the

unavoidable conclusion that the embryology of this interesting genus needs to be investigated more fully.

Tetrasporic embryo sacs of the *Adoxa*-type with more than eight nuclei occur only as abnormalities. *Ulmus americana* (Shattuck, 1905) is a well-known instance in which as many as twelve nuclei were seen in some cases. Some of the extra nuclei fused together with the polar nuclei and others organized as antipodal cells. Recently Billings (1933) has also noted some such cases in *Phoradendron villosum* and *P. flavescens*.

The following is a list of such plants in which the *Adoxa*-type of embryo sac has been reported. As will be seen from the preceding asterisks and "remarks", only a very few of these can now be regarded as correct:

Name of plant	Author	Remarks
	PIPERACEAE	
***Heckeria umbellata*	Johnson (1902)	In almost all of these the occurrence of a *Fritillaria*-type of embryo sac seems certain. See pp. 388 of this paper
**H. peltata*		
***Piper betel* var. *monoicum*	Johnson (1910)	
**P. medium*	Johnson (1902)	
***P. subpeltatum*	Palm (1915)	
**P. tuberculatum*	Fisher (1914)	
	JUGLANDACEAE	
***Carya amara*	Karsten (1902)	The author himself confesses that he had insufficient material. Langdon (1934), Woodroof (1928) and Shuhart (1932) have reported Normal-type in this genus
***C. tomentosa*		
***Juglans cordiformis nigra*	Karsten (1902)	Nawaschin & Finn (1913), Langdon (1934) and Nast (1935) report Normal-type in this genus
***J. regia*		
**Pterocarya fraxinifolia*	Karsten (1902)	This is also very uncertain
	ULMACEAE	
Ulmus americana	Shattuck (1905), Capoor (1937*b*)	—
U. hollandica belgica	Leliveld (1935)	—
U. Wilsoniana	Leliveld (1935)	These two species were not so fully studied
U. pumila pinnato-ramosa	Leliveld (1935)	
	SANTALACEAE	
Thesium intermedium	Modilewski (1928)	Guignard (1885) had, however, reported Normal-type in *T. divaricatum*
T. montanum	Schulle (1933)	
	LORANTHACEAE	
**Phoradendron villosum*	—	—
**P. flavescens* var. *macrophyllum*	Billings (1933)	—
	HYDNORACEAE	
**Hydnora africana*	Dastur (1922)	The stages figured in the paper are insufficient to justify *Adoxa*-type

	BALANOPHORACEAE[1]	
s *Balanophora dioica*	Ekambaram & Panje (1935)	The authors state that usually the development is of Normal-type, but there is sometimes a "tendency" towards *Adoxa*-type
	NYCTAGINACEAE	
s *Mirabilis jalapa* s *Oxybaphus nyctagineus*	Rocén (1927); only as abnormality	—
	AIZOACEAE	
***Mesembryanthemum pseudotruncatellum*	W. Schmid (1925)	Neumann (1935) calls this "eine aus verschiedenen Gründen recht zweifelhafte Angabe". See also Bhargava (1936)
	CARYOPHYLLACEAE	
**Stellaria media*	Gibbs (1907), P. C. Joshi (1936)	Rocén (1927) reported Normal-type. See pp. 393–4 of this paper for further information
	CRASSULACEAE	
***Sedum* sp.	D'Hubert (1896)	Mauritzon (1933, p. 21) calls this "fehlerhaft"
	SAXIFRAGACEAE	
***Philadelphus coronarius*	Van der Elst (1909)	Gäumann (1919) and Mauritzon (1933, p. 109) find Normal-type
	LEGUMINOSAE	
**Lupinus polyphyllus*	Guignard (1881)	Needs reinvestigation
***Medicago arborea*	Guignard (1881)	Reeves (1930) and Cooper (1936) find Normal-type
***Melilotus alba*	Young (1905)	Cooper (1933) finds Normal-type
	RUTACEAE	
s *Xanthoxylum alatum* s *X. Bungei*	Mauritzon (1935); only rarely	Usually Normal-type occurs
	LIMNANTHACEAE	
Limnanthes Douglasii	Stenar (1925*a*)	—
	TAMARICACEAE	
**Myricaria germanica*	Frisendahl (1912)	Needs reinvestigation; see remarks on p. 389 of this paper
**Tamarix tetrandra* **T. aestivalis* **T. africana* **T. gallica* **T. odessana* **T. pentandra*	Mauritzon (1936*a*)	Joshi & Kajale (1936) have reported *Fritillaria*-type in *T. dioica.* A reinvestigation is necessary
	CARICACEAE	
***Carica candamarcensis* ***C. chrysopetala* ***C. papaya* ***C. pentagona*	Heilborn (1921, 1928)	Kratzer (1918), and Agharkar & Banerji (1930) have found Normal-type in *C. papaya*
	CACTACEAE	
***Phyllocactus* sp.	d'Hubert (1896)	On the basis of their experience of some other Cactaceae, both Mauritzon (1934*c*) and Neumann (1935) regard this as quite incorrect

[1] The parthenogenetic species are not listed here.

	MYRTACEAE	
*_Eugenia jambos_ *_E. bancana_	Pijl (1934)	See remarks on p. 395 of this paper
	OENOTHERACEAE	
**_Trapa natans_	Gibelli & Ferrero (1891)	Ishikawa (1918) saw megaspore tetrads and Maheshwari (unpubl.) in _T. bispinosa_
	ARALIACEAE	
*_Aralia spinosa_	Ducamp (1902)	Needs reinvestigation
	PLUMBAGINACEAE	
Armeria alpina	—	In view of Haupt's (1934) work, a reinvestigation of the other Plumbaginaceae is very desirable
A. plantaginea	Dahlgren (1916)	
A. vulgaria	—	
s _Ceratostigma plumbaginoides_[1]	Dahlgren (1916)	—
Plumbago capensis	Haupt (1934)	—
Statice bahusiensis	—	—
S. Gmelini	Dahlgren (1916)	—
S. macroptera	—	—
	OLEACEAE	
**_Forsythia suspensa_	Billings (1901)	Andersson (1931, p. 52) has shown that Normal-type occurs
	SOLANACEAE	
**_Solanum muricatum_	Nanetti (1912)	See remarks by Rees-Leonard (1935)
**_S. tuberosum_	Young (1923)	Corrected by Rees-Leonard (1935), who finds Normal-type
	GESNERIACEAE	
*_Rhytidophyllum crenulatum_	Cook (1907)	Needs reinvestigation
	ACANTHACEAE	
*_Acanthus ilicifolius_	Karsten (1891)	Needs reinvestigation, since the author is himself uncertain
	CAPRIFOLIACEAE	
*_Sambucus racemosa_	Lagerberg (1909)	Jönsson (1879–80) reported Normal-type
	ADOXACEAE	
Adoxa moschatellina	Jönsson (1879–80), Lagerberg (1909)	—
	COMPOSITAE	
s _Leontodon hispidus_	Bergman (1935)	Usual development is normal, but in some cases the walls separating the megaspore nuclei dissolved and there was only one more division
	TYPHACEAE	
**_Typha latifolia_	Schaffner (1897_b_)	Dahlgren (1918) showed this to be incorrect and found Normal-type
	ALISMATACEAE	
**_Alisma plantago_	Schaffner (1896)	Dahlgren (1916) & Johri (1936_b_) have shown that the development is of the _Allium_-type

[1] An 8-nucleate condition was seen in only one case.

	BUTOMACEAE	
**Limnocharis emarginata*	Hall (1902)	Johri (1936*b*) has given a different interpretation of Hall's figures and believes that *Allium*-type occurs here.
	PALMACEAE[1]	
**Cocos nucifera*	Quisumbing & Juliano (1927)	Earlier stages have not been fully investigated
**Chamaerops humilis*	Gioelli (1930*b*)	
	ARACEAE	
***Acorus calamus*	Mücke (1908)	Jüssen (1928) reports Normal-type
**Anthurium violaceum* var. *leucocarpum*	Campbell (1905)	Needs reinvestigation
***Richardia africana*	Gow (1913)	Michell (1916) finds Normal-type
***Zantedeschia aethiopica*	Gow (1913)	Michell (1916) finds Normal-type
	LEMNACEAE	
**Lemna minor*	Caldwell (1899)	Jönsson (1879–80) reported *Allium*-type in *L. trisulca*
	BROMELIACEAE	
s *Tillandsia usneoides*	Billings (1904)	In a very few abnormal cases no walls were seen after reduction division
	CYNASTRACEAE	
***Cynastrum Johnstoni*	Th. C. E. Fries (1919)	Stenar (unpubl.) has found Normal-type in *C. Goetzeanum*, and Nietsch (unpubl.) in some other species of the same genus
	LILIACEAE	
**Aloe arborescens* **A. caesia* **A. ciliaris* **A. Todari* var. *praecox* **A. Varvari*	Gioelli (1930*a*)	Schnarf & Wunderlich (unpubl.) find Normal-type in the species of *Aloe* they have investigated. Gioelli's Fig. 9 of *A. caesia* probably shows a T-shaped tetrad of megaspores that has been misinterpreted as a 4-nucleate embryo sac due to bad fixation
Camassia Quamash	Leffingwell (1930)	Only a few stages have been figured by the author
***Convallaria majalis*	Wiegand (1900)	Schniewind-Thies (1901) found Normal-type
**Erythronium albidum* **E. americanum*	Schaffner (1901)	A reinvestigation may reveal that the *Fritillaria*-type occurs here
**Gagea lutea*	Stenar (1927)	Romanov (1936) has demonstrated *Fritillaria*-type in three species of this genus and thinks that its occurrence is almost certain in *G. lutea* also
**Medeola virginica*	McAllister (1909)	Figs. 40 and 41 of the author admit interpretation of *Fritillaria*-type
***Majanthemum canadense*	McAllister (1914)	Stenar (1934) has found *Peperomia*-type in *M. bifolium*

[1] As pointed out by Schnarf (1931, p. 280) a reinvestigation of the Palmaceae is very desirable. The lack of agreement among the various authors who have worked on this family seems to be due to the earlier development taking place while the inflorescence is still inside the spathe—a fact often realized by the investigator only *after* he has finished his collections and sectioned the material he had.

**Smilacina sessilifolia*	McAllister (1914)	See remarks by Stenar (1934) who suspects that a 16-nucleate embryo sac of the *Peperomia*-type is present here also, as in *Majanthemum*
**S. stellata*	McAllister (1909, 1914)	
***Tulipa gesneriana*	Ernst (1901)	Figs. 13 and 14 of Ernst show a 'secondary 4-nucleate stage"; later Bambacioni & Giombini (1930) demonstrated that *Fritillaria*-type does occur
**T. celsiana*	Guignard (1900	In the light of Bambacioni's work a detailed reinvestigation of all these species seems to be imperative
**T. humilis*	Newton (1926)	
**T. kalpakowskiana*	Newton (1926)	
**T. silvestris*	Guignard (1900)	
	AMARYLLIDACEAE	
**Amaryllis belladonna*	Schlimbach (1924)	The account is very meagre and there are absolutely no illustrations in this paper
**Buphane disticha*	Schlimbach (1924)	
**Cooperia Drummondii*	Church (1916)	Needs reinvestigation
***Crinum asiaticum*	Schlimbach (1924)	Stenar (1925*b*) & Tomita (1931) report *Allium*-type in other species of this genus
**Cyrtanthus sanguineus*	Farrel (1914)	The author says it "seems" to follow the *Adoxa*-type. Stiffler (1925) has shown that Normal-type occurs in *C. parviflorus*
**Nerine curvifolia*	Schlimbach (1924)	Needs reinvestigation
	ZINGIBERACEAE	
***Costus* sp.	Humphrey (1896)	Both of these reports seem to be incorrect. Banerji & Venkateswarlu (1936) have demonstrated a normal tetrad of megaspores in *C. speciosus*
***C. igneus*	Mauritzon (1936*b*); "very probable"	

Plumbagella-type

Dahlgren, in 1916, reported that in *Plumbagella micrantha*, *Plumbago capensis*, *P. pulchella*, *P. zeylanica* and *Ceratostigma plumbaginoides*,[1] only two divisions intervene between the megaspore mother cell and the egg and these are the obviously necessary reduction divisions. Of the four free nuclei thus formed after reduction, the micropylar pair gives rise to the egg and upper polar nucleus and the chalazal pair forms the single ephemeral antipodal and the lower polar nucleus.

As mentioned before, Haupt (1934) has reinvestigated *Plumbago capensis* very fully and the radical differences between his account and that of Dahlgren (1916) raise some doubts about the reliability of the earlier observations.[2] Dr Dahlgren (as mentioned in a private communication to the writer) is investigating some species himself

[1] In this case Dahlgren observed one 8-nucleate embryo sac also.
[2] See also the remarks by Schnarf (1936, p. 577).

and further opinion may therefore be deferred till his latest observations have been published.

The embryo sacs of *Aglaonema simplex* and *A. modestum*, usually included under the *Adoxa*-type, may with greater propriety be considered here. According to Campbell's (1912) statement, the four megaspore nuclei are arranged in the usual manner, but only one of

Name of species	Megaspore mother cell	I division	II division	III division	Mature embryo sac	Remarks
F. splendens (less than 50% cases)						Embryo sac 8-nucl. (*ADOXA*-type)
F. splendens (commonest condition)						Embryo sacs 6-nucl. (reduced *ADOXA*-type). 2 syn. and 2 antipodals always present, but sometimes only 1 polar nucleus and an egg; sometimes 2 "presumable" polars and *no egg*
F. splendens (only rarely); *F. burragei*; *F. peninsularis* ("always")						Embryo sac 5-nucl. After 4-nucl. stage, only one micropylar nucl. divides, forming the 2 synergids. (*AGLAONEMA*-type.)

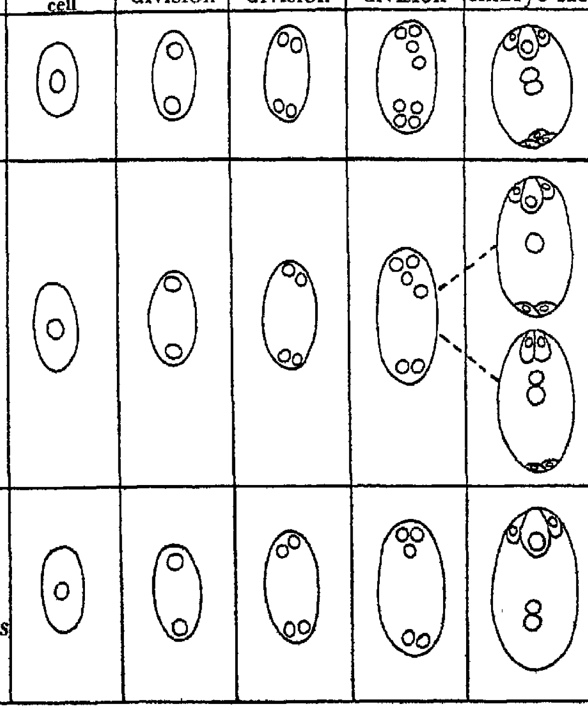


Fig. 15. Diagrams prepared to illustrate the range of variation reported to occur in *Fouquiera* by Johansen (1936).

the micropylar nuclei divides again and this division gives rise to the two synergids; the second *undivided* nucleus functions as the egg and the two chalazal nuclei as polars. If these observations are correct, one of the megaspore nuclei itself functions as the egg, which is therefore removed from the mother cell *by only two divisions*. The third division concerns only the production of the two synergids and therefore this type of embryo sac may be considered as a modification of the *Plumbagella*-scheme. As Michell (1916, p. 333) remarked, however, "the obstacle in the way of accepting Campbell's view is that he has never been able to demonstrate the supposed nuclear division".

Very recently Johansen (1936) has reported the same type of development in three species of *Fouquiera: F. splendens, F. peninsularis* and *F. burragei* (see Fig. 15). In some cases (only in *F. splendens*)

it was found that both the micropylar nuclei divided instead of only one, resulting in 6-nucleate embryo sacs with two different types of organization, and in still others all four has divided to form 8-nucleate embryo sacs (*Adoxa*-type). On the other hand Mauritzon (1936*a*, p. 95) who has also studied *F. splendens* (independently and without knowledge of Johansen's work) says that the development follows the Normal-type. The differences between these two observers point to the need of a reinvestigation.

In conclusion it may be said that the *Plumbagella*-type, which remained unquestioned for about 20 years (largely because no other embryologist besides Dahlgren studied this family), must now be regarded as uncertain. The range of variation reported in *Aglaonema* and *Fouquiera* is possible but not probable and both demand a fresh study.

HOMOLOGIES OF THE ANGIOSPERM EMBRYO SAC

This is a question whose solution does not seem to be possible, at least at present. There are three main views in the field:

(1) That the embryo sac of angiosperms is derived by reduction from that of some hypothetical conifer and now consists of only two archegonia without any prothallial tissue (Porsch, 1907). The synergids (=neck cells), egg, and upper polar nucleus (=ventral canal nucleus) are supposed to constitute the first archegonium, while the three antipodal cells and lower polar nucleus form the second but now functionless archegonium (for explanation see Fig. 16).

(2) That one synergid (=a ventral canal nucleus) and the egg, both of which are sister cells, constitute one archegonium, while the second synergid and the upper polar nucleus constitute a second archegonium. The remaining nuclei are to be regarded as prothallial. Prof. Schürhoff (1928), who put forward this view, thinks that this also gives a rational explanation of "double fertilization", for both the archegonia are fertilized, although only one happens to give rise to the embryo.

(3) That all the nuclei of the embryo sac are potential eggs, although only one of them produces an embryo. This idea was inspired by the discovery of certain features in the ovule and embryo sac of *Gnetum*, which seemed to show a strong resemblance to angiosperms (see literature cited in Thompson, 1916).

The first and the second view both depend on certain assumptions which are contrary to one another. While Porsch thinks that the synergids are sister cells, Schürhoff contends that one synergid

nucleus is sister to the egg and the second to the upper polar nucleus. Leaving alone certain abnormal cases (like *Peperomia* spp., which do not yet seem to have been fully investigated from this point of view) there is an overwhelming evidence in favour of Porsch's view. Several recent workers have reported that the synergids are formed

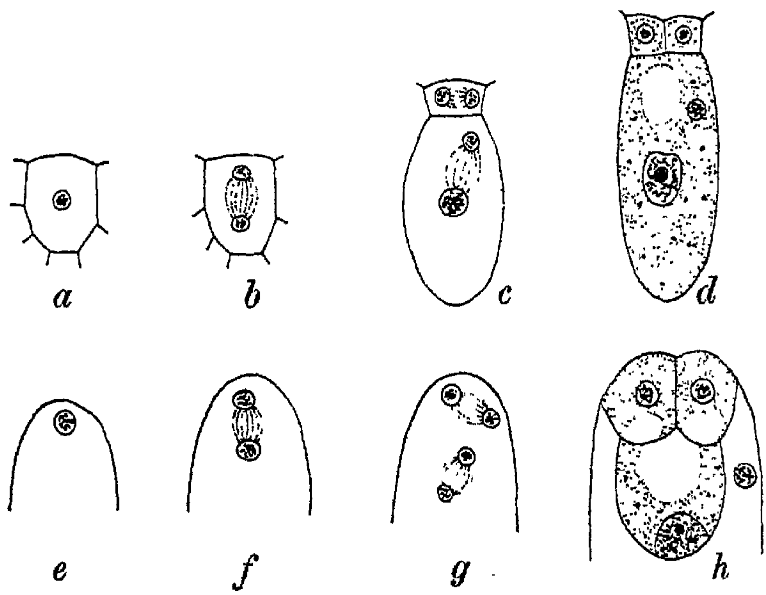


Fig. 16

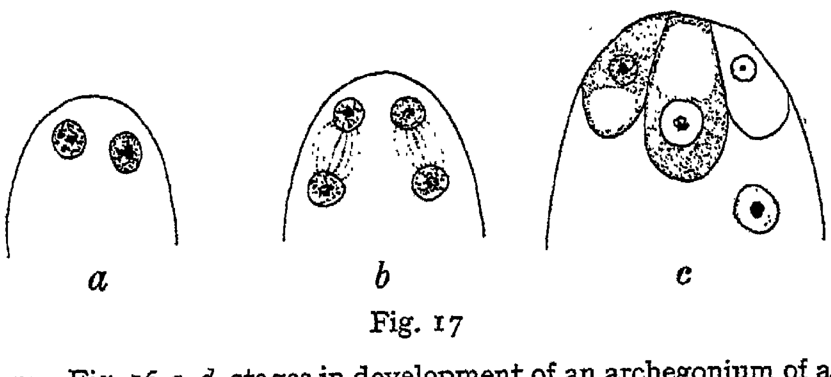


Fig. 17

Figs. 16, 17. Fig. 16 *a–d*, stages in development of an archegonium of a gymnosperm; *e–h*, stages in development of upper half of angiosperm embryo sac (after Porsch, 1907). Fig. 17 *a–c*. Diagrams of upper half of angiosperm embryo sac, prepared to illustrate Schürhoff's view. In (*c*) the egg and one synergid (shaded) represent "one archegonium", while the other synergid and polar nucleus (unshaded) represent the "second archegonium".

from the division of one nucleus, and the egg and upper polar nucleus from another nucleus of the 4-nucleate stage.[1]

There are, however, other difficulties in accepting Porsch's view. What reason is there for the ventral canal nucleus (upper polar

[1] Only a few instances may be cited here: *Hartmannia tetraptera* (Johansen, 1929), *Boerhaavia diffusa* (Maheshwari, 1929), *Ionopsidium acaule* (Corti, 1930), *Dysoxylum ramiflorum* (Paetow, 1931), *Urginea indica* (Capoor, 1937*a*). See also Langlet (1927).

nucleus) to leave its position above the egg and come down into the centre of the embryo sac to fuse with another nucleus from the chalazal end which is also the ventral canal nucleus of a second archegonium? We have absolutely no records of such plants which may be visualized as providing a real series of intermediate stages between Porsch's hypothetical gymnospermic ancestor and the modern type of angiosperm.

A detailed discussion of the third view does not seem to be possible. While formerly it was thought that the many-nucleate embryo sacs of *Pandanus*, *Peperomia*, etc. represented a transition from the condition in *Gnetum* to that in the typical angiosperm, no one seriously holds the view at present. The number of abnormal embryo sacs discovered in angiosperms is very small indeed and those, that have been found, occur in the most primitive as well as the most advanced families.

The angiosperms which we see to-day cannot be compared with any other living group. It is true that the Gnetales show certain significant resemblances in the organization of the ovule and in the development of the anther and female gametophyte, but modern trend of thought is in favour of regarding this only as an instance of parallel development. Fossil plants are seldom so well preserved as to give an insight into the structure of the gametophytes, but results of considerable value will be obtained when such material *is* found (Florin's recent work on Cordaitales is an instance of this kind). The question continues to be as baffling as before, but we believe that detailed investigations on the morphology of the Gnetales on the one hand and the more primitive angiosperms on the other will yield results that will eventually help in the solution of the problem.[1] Dr Johri's (1936*a*) recent discovery of the occurrence of pollen grains in the stylar canal and ovary of *Butomopsis* is an instance of the surprises that lie in wait for us.

[1] Prof. W. P. Thompson, who made a very thorough investigation of the embryology of several species of *Gnetum*, writes (1916, p. 176): "the sum of the evidence from all sides seems to lead to the conclusion that Angiosperms are phylogenetically related to Gnetales. This does not mean that any modern member of the Gnetales represents the type from which Angiosperms were derived but that the ancestors of Angiosperms were not far removed from the genus *Gnetum*."

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THE ANAEROBIC METABOLISM OF THE MOULD FUNGI IN RELATION TO CITRIC ACID FORMATION

By T. G. TOMLINSON
Botanical Department, University of Manchester

(With 8 figures in the text)

Introduction

Although a considerable amount of work has been done on the organic acid production of the mould fungi the initial processes by which the sugar present in the culture solution is broken down are not clearly understood. Two important hypotheses of organic acid synthesis by the moulds should be mentioned. First, according to the Chrząszcz-Tiukow (1930) hypothesis, the glucose molecule is first broken down to acetaldehyde with subsequent oxidation of the acetaldehyde to citric acid. Secondly, according to the Franzen-Schmitt (1925) hypothesis, a direct oxidation of the glucose molecule takes place in which gluconic acid is produced. This is converted successively to saccharic acid, diketoadipic and citric acids. The citric acid is then commonly supposed to be oxidized finally to oxalic acid and carbon dioxide. The production of citrates by *Aspergillus niger* and various Penicillia supplied with acetates as the sole external source of carbon is cited in support of the first hypothesis. The evidence of Challenger *et al.* (1927) in support of the second hypothesis consists of the discovery of saccharic acid and citric acid in cultures of *A. niger* grown on calcium gluconate and the observation that cultures supplied with saccharates form citrates. Müller's (1928) discovery of the enzyme glucose-oxidase which oxidizes glucose to gluconic acid in *A. niger* lends support to the Franzen-Schmitt hypothesis. If the first hypothesis is correct the zymasic activity of the fungus would have to be great enough to account for the relatively high rate of formation of organic acids and carbon dioxide produced by the fungus.

Our present knowledge of the part played by the zymase system in the metabolism of the mould fungi is very incomplete and very contradictory statements appear in the literature. Wehmer (1907)

stated that with one exception the mould fungi do not produce alcohol. Euler & Myrbäck (1924) were unable to find cozymase in *Penicillium glaucum*. Müller (1929) found that zymase is present in *Aspergillus niger* in only very small quantities. Raistrick and co-workers (1931), on the other hand, measured the volatile neutral compounds produced by many species of moulds under semi-anaerobic conditions and concluded that certain groups of moulds are alcohol producers and others are not: some of the non-alcohol producers show active acid formation. Kostytschew (1907) found that a culture of *A. niger* when grown on sugar produced alcohol and carbon dioxide anaerobically in the theoretical ratio for alcoholic fermentation ,but Kostytschew & Afanassjewa (1921) found that when *A. niger* was grown on peptone it was unable to produce alcohol anaerobically even when supplied with sugar. It was deduced from this that cultures grown on sugar were able to produce zymase and were, therefore, capable of "sugar respiration". Those grown on peptone were unable to produce zymase and had, therefore, to rely on another type of respiration which he termed "protein respiration".

The results described in the present paper show that of a considerable number of fungi examined all carry our normal zymasic fermentation under anaerobic conditions. The data regarding carbon dioxide output in air and nitrogen show, however, that there is considerable doubt as to the ability of the zymase system to bring about the observed high rates of aerobic sugar breakdown and acid formation in many of the species examined. Kostytschew's claim of the distinctness of "sugar" and "protein respiration" has been examined, but his claims are not substantiated and it has not been found possible to produce a "zymase-free" *Aspergillus* by culture on special media. It had, of course, been intended to examine the citric acid production (if any) of "zymase-free" cultures. The bearing of these results on the "chemismus" of citric acid production in the mould fungi is discussed.

Experimental results

Group I. Products formed by various species of mould fungi during anaerobiosis

The methods of growing the experimental cultures and of obtaining the air rate of respiration under sterile conditions were the same as those used by Bennet-Clark & La Touche (1935).

Exp. A1. *A. niger* (SI 133C) was grown on 200 c.c. 5% glucose with the inorganic salts of Czapek's solution in a 500 c.c. bolt-head flask. At the start of anaerobiosis the culture was 8 days old. The original culture solution was withdrawn from the flask and the mycelium washed twice with sterilized water. 200 c.c. 5% glucose solution was then drawn into the flask. The method of establishing anaerobic conditions within the flask quickly was to evacuate the flask three times to a pressure of 5 cm. mercury with subsequent admission of nitrogen previously freed from traces of oxygen by Kautsky & Thiele's (1926) method. After 2 days' anaerobiosis the carbon dioxide produced was obtained by evacuating the flask through an absorption tube containing sodium hydroxide. The alcohol obtained by distillation of an aliquot part of the culture solution was oxidized to acetic acid and determined titrimetrically (Thomas, 1925).

The rates of respiration, alcohol production, etc. are recorded in terms of mg. of carbon evolved in the form of carbon dioxide, alcohol, etc. per hr. 48·2 mg. C in carbon dioxide and 97·0 mg. C in alcohol were formed in 48 hr. The dry weight of the fungus was 1·67 g. Hence the ratio of carbon-dioxide carbon to alcohol carbon is 0·496 and the average rate of anaerobic respiration is 1·05 mg. C/g./hr. The rate of respiration in air before the period of anaerobiosis was 6·8 mg. C/g./hr. The ratio of nitrogen rate to air rate of respiration (NR/OR) is, therefore, 0·15. The data of this and similar experiments with other species of moulds are given in Table I. The ratio of carbon-dioxide carbon to alcohol carbon is approximately 0·5 in all the species examined. All the species investigated from types like *A. niger* and *A. oryzae* which are well-known alcohol producers to *A. glaucus* and *Penicillium citrinum* which are said to be non-producers of alcohol are found to be capable of alcoholic fermentation. However, zymase appears to be very unevenly distributed among the species examined here, and there seems to be no uniform relationship between the air rate of respiration and the nitrogen rate.

From the Chrząszcz-Tiukow hypothesis it might be expected that these species of fungi with a relatively high zymase content would also be active citric acid producers. To determine whether active zymasis accompanies active citric acid production the following experiments were carried out. It will be sufficient to describe one of these and reference may be made to Table II for the results of the twelve experiments.

TABLE I. *Rates of anaerobic carbon dioxide and alcohol formation by various species of moulds*

Exp. no.	Species	Catalogue no.	Age days	Dry weight g.	CO_2 carbon mg.	Alcohol carbon mg.	CO_2 carbon / Alcohol carbon	NR mg. C/g./hr.	OR mg. C/g./hr.	NR/OR
A 1	*Aspergillus niger*	SI 133C	8	1·67	48·2	97·0	0·496	1·05	6·8	0·15
A 2	*A. niger*	M 1	5	0·75	86·3	157·0	0·55	2·4	10·3	0·23
A 3	*A. oryzae*	SI 202	7	0·26	161·5	374·0	0·43	8·65	18·3	0·47
A 4	*A. tamarii*	SI 28	5	0·70	56·1	99·5	0·565	1·7	11·3	0·15
A 5	*A. flavipes*	SI 175*a*	8	0·54	17·3	33·6	0·515	0·66	5·8	0·11
A 6	*A. candidus*	SI 158	7	0·40	18·0	31·6	0·57	0·27	3·9	0·079
A 7	*A. ochraceus*	SI 162	7	0·34	23·6	374·0	0·63	0·97	14·4	0·067
A 8	*A. glaucus*	M 4	10	0·37	5·2	11·4	0·46	0·07	11·1	0·006
A 9	*Penicillium divaricatum*	SI 225	6	0·39	52·1	106·0	0·49	1·4	12·8	0·11
A 10	*P. sanguifluus*	M 7	4	0·21	7·92	12·65	0·62	0·79	26·6	0·03
A 11	*P. candidofulvum*	SI 85	8	0·25	3·72	8·5	0·44	0·21	4·0	0·05
A 12	*P. citrinum*	SI 68	10	0·45	21·2	40·5	0·52	0·33	16·6	0·02

TABLE II. *Amounts of free citric or oxalic acid produced by various species of moulds*

Exp. no.	Species	Catalogue no.	Age days	Free acid c.c. 0·218 *N* NaOH	Oxalic acid	Citric acid	Dry weight g.	g. free acid / g. dry weight	Zymase activity
B1	*Aspergillus niger*	SI 133C	5	19·17	+++	+	0·65	0·29 oxalic	++
B2	*A. niger*	M1	7	6·64	+++	+	0·55	0·118 oxalic	+++
B3	*A. oryzae*	SI 202	8	2·64	0	0	0·40	?	++++
B4	*A. tamarii*	SI 28	5	0	0	+	0·43	0	+++
B5	*A. flavipes*	SI 175*a*	7	0	0	+	0·68	0	+
B6	*A. candidus*	SI 158	9	0	0	0	0·025	0	+
B7	*A. ochraceus*	SI 162	7	0	0	0	0·74	0	++
B8	*A. glaucus*	M4	9	0	0	0	0·06	0	+
B9	*Penicillium divaricatum*	SI 225	7	15·0	0	+++	0·53	0·396 citric	+++
B10	*P. sanguifluus*	M7	8	8·55	0	+++	0·44	0·272 citric	+
B11	*P. candidofulvum*	SI 85	9	0	0	+	0·174	0	+
B12	*P. citrinum*	SI 68	8	1·78	0	+++	0·45	0·0054 citric	+

Exp. B1. *Aspergillus niger* (M1) was grown on 100 c.c. 5% glucose Czapek solution in a 250 c.c. conical flask fitted with a loose cotton-wool bung. Conidium formation was at its maximum when the culture was 7 days old. The culture solution was now analysed qualitatively for oxalic and citric acid and also the amount of free acid produced by the fungus was determined by titrating with 0·218*N* NaOH and subtracting the blank titration of the original culture solution. The amount of free acid in the culture solution corresponded to 6·64 c.c. 0·218*N* NaOH. A large precipitate of calcium oxalate was obtained on adding calcium acetate solution, but only a faint cloudiness due to pentabromacetone was obtained by Kunz's (1925) method of determining citric acid. This indicated that most of the free acid must be oxalic acid. The dry weight of the fungus was 0·55 g. so that the net amount of oxalic acid produced by *A. niger* (M1) in 7 days was 0·118 g./g. dry wt. The relative zymasic activity of the various species is indicated by plus signs in the last column of Table II obtained from Exps. A1–A12 (see Table I, column of NR).

Although *A. oryzae* has the largest zymasic activity of all the species examined it will be seen from Table II that it produced a relatively small amount of titratable acid, the nature of which was not determined. Both strains of *A. niger* contain moderately large amount of zymase and also produce large amounts of oxalic acid. On the other hand it will be seen that *Penicillium citrinum* and *P. sanguifluus* both contain small amounts of zymase but produce large amounts of citric acid. No correspondence between zymasic activity and citric acid formation such as would be expected on the basis of the Chrząszcz-Tiukow hypothesis can be deduced from these data.

Group II. The anaerobic respiration of peptone cultures of Aspergillus niger (SI 133C)

Peptone cultures of *A. niger*, according to Kostytschew, are zymase-free and on the basis of the Chrząszcz-Tiukow hypothesis should fail to produce citric and oxalic acids from sugar.

Exp. C1. *A. niger* (SI 133C) was grown as in Exp. A1 with the exception that the 200 c.c. of culture solution contained 6 g. peptone instead of glucose, together with the inorganic salts of Czapek's solution. The spores germinate quickly on this medium, but the fungus develops only a thin mycelium and consequently the rate of

respiration remains at a relatively low level compared with that of glucose cultures. By the 10th day a uniform fungus mat covered the surface of the culture solution. The methods of establishing anaerobiosis and measuring the amounts of carbon dioxide and alcohol were the same as before. The duration of the anaerobic period was 10 days. The dry weight of the fungus was only 0·127 g. and in 10 days 8·1 mg. carbon in carbon dioxide and 16·2 mg. carbon in alcohol were formed in nitrogen. The air rate of respiration just prior to the period of anaerobioses was 16·0 mg. C/g./hr. The average nitrogen rate of respiration was 0·27 mg. C/g./hr. The data of this and three similar experiments are given in Table III.

TABLE III. *Anaerobic carbon dioxide and alcohol production of peptone cultures of* A. niger (SI 133C)

Exp. no.	Age days	Dry weight g.	CO_2 carbon mg.	Alcohol carbon mg.	$\frac{CO_2 \text{ carbon}}{\text{Alcohol carbon}}$	NR mg. C/g./hr.	OR mg. C/g./hr.	$\frac{NR}{OR}$
C 1	10	0·127	8·1	16·2	0·50	0·27	16·0	0·017
C 2	10	0·198	8·6	17·6	0·49	0·18	10·0	0·018
C 3	10	0·20	6·8	14·2	0·49	0·14	11·0	0·013
C 4	10	0·19	7·2	14·8	0·48	0·16	10·5	0·015
A 1	Glucose culture for comparison					1·05	6·8	0·15

In all the four experiments, C 1–C 4, alcohol was produced anaerobically although in very small quantities. It is likely that such small quantities would be undetected in Kostytschew's experiments which were of much shorter duration. On the other hand the ratio of carbon-dioxide carbon to alcohol carbon is about 0·5 and is that of normal alcoholic fermentation. It is true, however, that the zymasic activity of peptone cultures is relatively much smaller than the zymasic activity of glucose cultures, as will be seen in Table III by comparing the nitrogen rates of respiration of the peptone cultures C 1–C 4 with those of the glucose culture A 1.

Group III. The course of respiration during alternate periods in air and nitrogen

Exp. F 1. *A. oryzae* (SI 202) was grown as in Exp. A 1. The CO_2 readings were at first taken every day but later at about 4-hourly intervals. At the 180th hr. anaerobiosis was established within the culture flask by the technique described in A 1 in about 10 min. Nitrogen was then passed through the culture flask at a rate of 1 l./hr. and the first reading of nitrogen respiration (NR) was taken 70 min.

after the start of anaerobiosis. The fungus remained on the original nutrient solution during the whole of the experiment. It has been shown by experiments not described in this paper that subjecting the culture to a pressure of about 5 cm. mercury for 15 min. has no effect on the subsequent respiration and behaviour of the fungus. After the first 2 hr., 4-hourly readings of the CO_2 output in nitrogen were taken over a period of 44 hr. After this the course of respiration was noted for a further 3 days in air. The results of this experiment are expressed graphically in Fig. 1. The rates of CO_2 production are given as ordinates in mg. C/hr./culture and time in hours is plotted on the horizontal axis. The dotted lines represent the idealized respiration curves based on which an analysis can be made on the

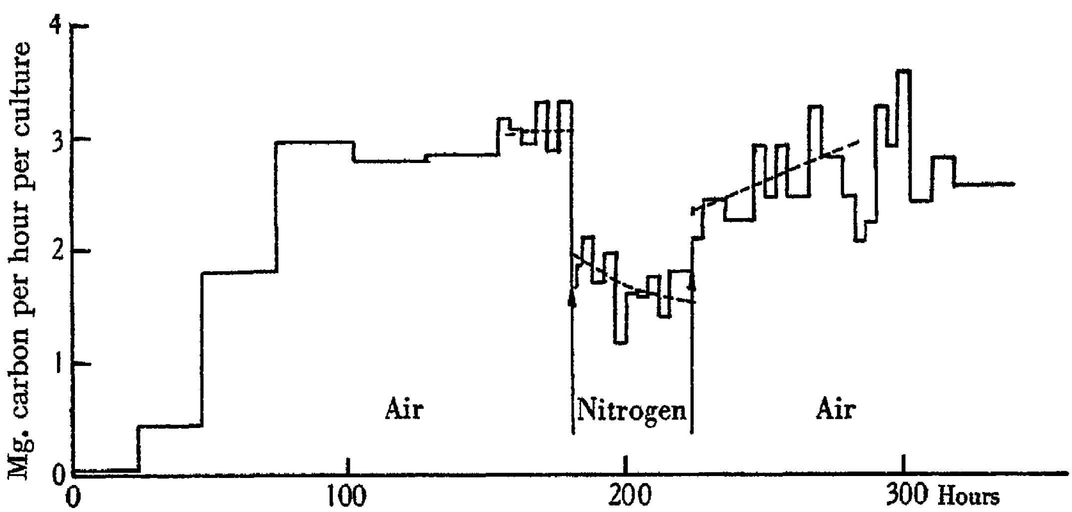

Fig. 1. Experiment F 1. *A. oryzae.*

lines of that developed by Blackman (1928) for the data on the respiration of apples obtained by Parija. The evaluation of the initial NR is very precarious. The record on the whole suggests that the initial NR is that obtained by producing back the dotted line to the hour of nitrogen supply. Initial NR/final OR, therefore, is $\frac{1\cdot 95}{3\cdot 05} = 0\cdot 64$. On returning to air there is no sharp transitional phase and the ratio final NR/initial OR is quite indeterminate. The record is at least consistent, however, with the view that this value is also about 0·64. Though these very rough provisional values characteristic of *A. oryzae* are lower than unity (as compared with the apple data in which values higher than unity are obtained) they are not inconsistent with the presence of a system similar to that postulated for apple tissue. They indicate a rate of glycolysis at the time of

transition equal roughly to 1·9 times OR and thus in terms of the Blackman schema a rate of oxidative anabolism 0·9 times OR.

Exp. F2 was similar to the previous experiment except that *A. tamarii* (SI 28) was the species used and also an exactly similar culture was started with fructose instead of glucose as the source of carbon. The data are given in Figs. 2 and 3 with the dotted lines added to facilitate interpretation of the results. It will be seen from these curves that there is no appreciable difference in behaviour between the glucose and the fructose cultures. Both cultures grew rapidly and by the 100th hr. were respiring at a rate of about

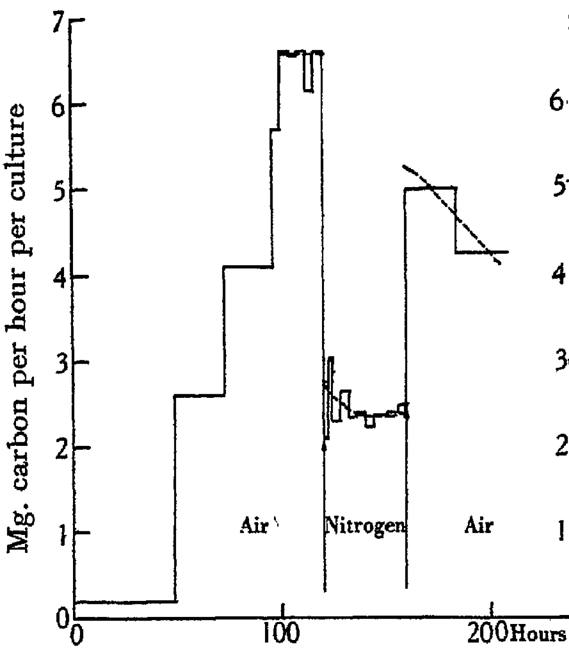

Fig. 2. Experiment F2. *A. tamarii* on glucose.

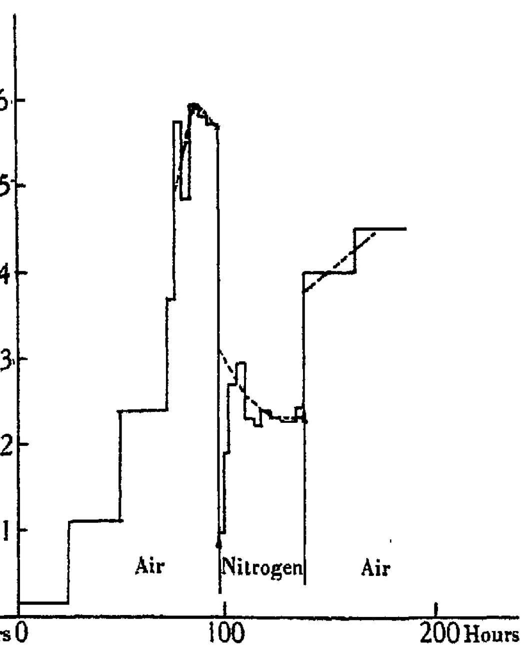

Fig. 3. Experiment F2. *A. tamarii* on fructose.

6 mg. C/hr. For the glucose culture (Fig. 2) initial NR/final OR = 0·41 and the rate of glycolysis would be 1·3 times OR. The corresponding values for the fructose culture (Fig. 3) are 0·54 and 1·7 respectively.

Exp. F3 was carried out in the same way with *A. niger* (SI 133C). It will be seen from Fig. 4 that the CO_2 output in nitrogen decreased steadily during the first 18 hr. anaerobiosis to a steady value at about 0·8 mg. C/hr. Unfortunately it is not clear whether this is due to the technique, i.e. to CO_2 coming out of solution or whether it is due to progressive decrease in actual rate of CO_2 production by the fungus. On these two bases the values of initial NR/final OR could be chosen as being either 0·38 indicating a very low rate of oxidative anabolism or as 0·20. Values of NR/OR less than 0·33 necessitate some reconsideration of the basis of treatment of these results. The transition

from nitrogen to air is complicated by the physical factors of the resaturation of the culture solution which contains in solution some 2 c.c. CO_2 (1 mg. CO_2 carbon) and the initial air rate is thus obscured.

Exp. F4. Another culture of *A. niger* (SI 133C) was investigated which behaved in some ways similarly to F3. From Fig. 5 it will be

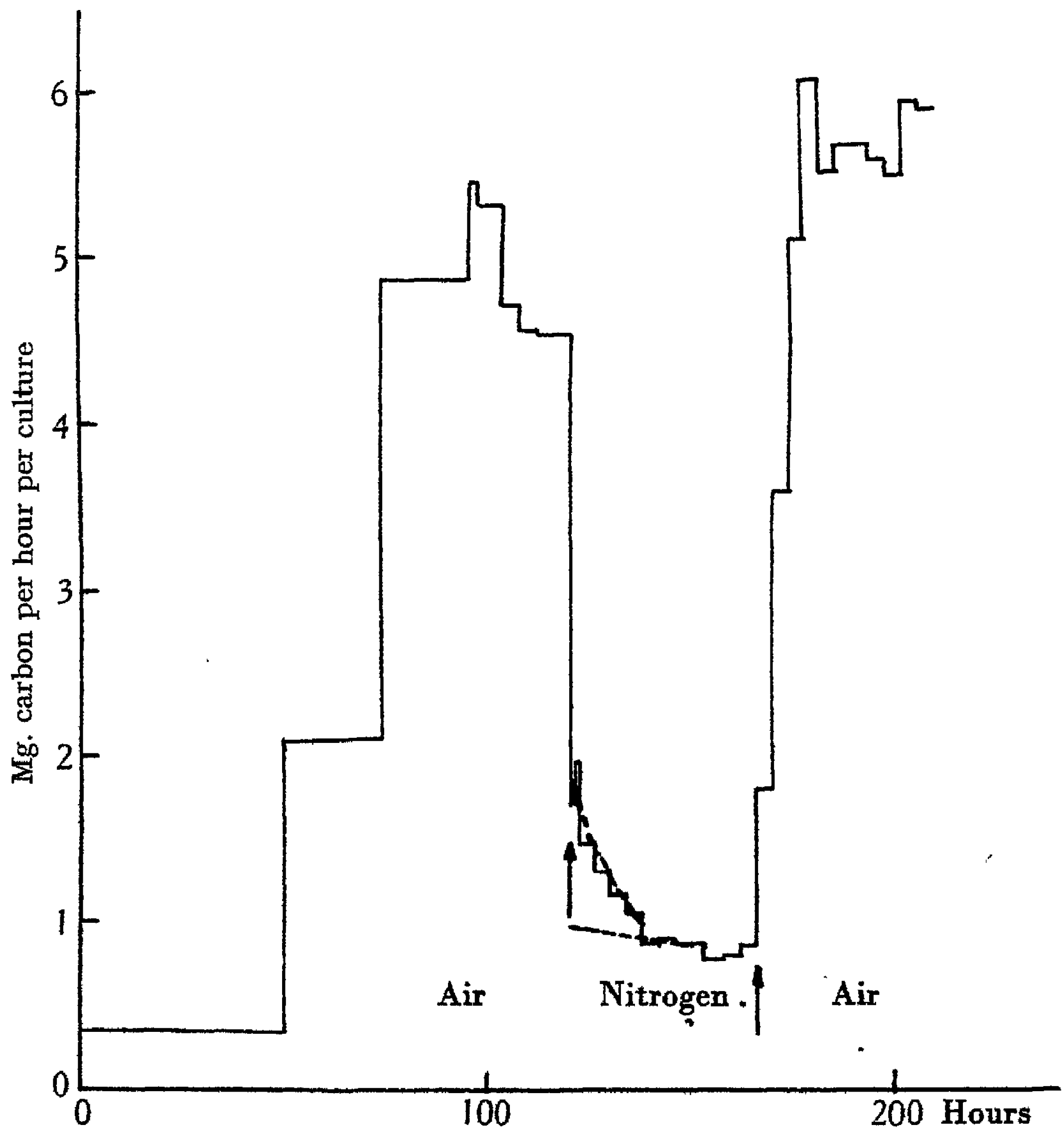

Fig. 4. Experiment F3. *A. niger*

seen that the nitrogen rate fell to a steady value much below that of F3. Using the two constructional lines for the initial NR the values of initial NR/final OR 0·077 and 0·029 are obtained. The transition from nitrogen to air suggests a value of about 0·15 for the ratio of initial NR/final OR, but this treatment cannot properly be applied to records of this type.

Exp. F5. *Penicillium sanguifluus* behaved in a similar way to

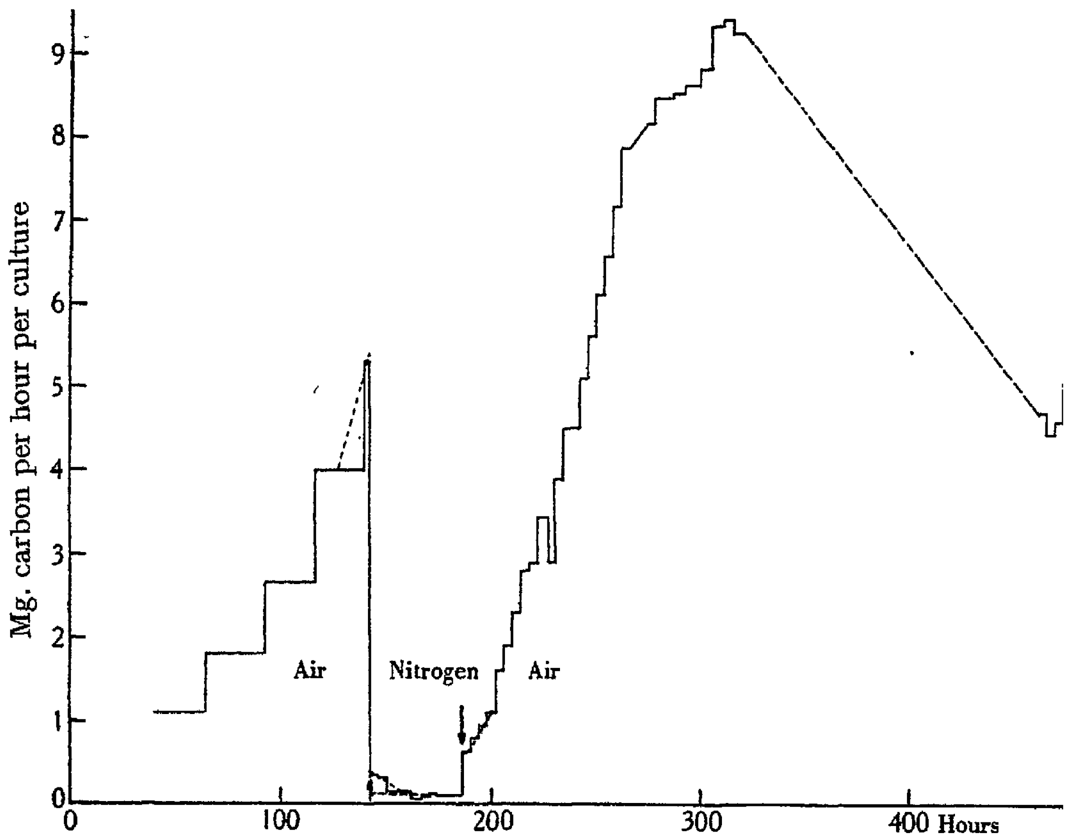

Fig. 5. Experiment F 4. *A. niger.*

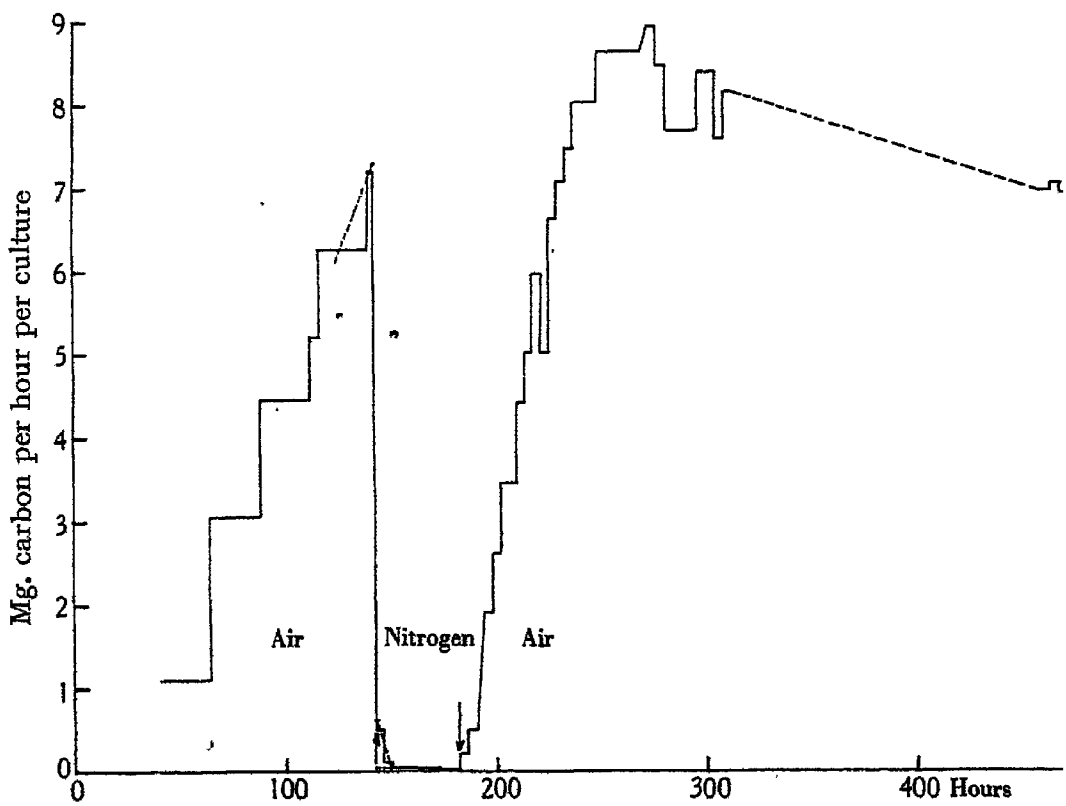

Fig. 6. Experiment F 5. *P. sanguifluus.*

F4. From Fig. 6 the numerical values for initial NR/final OR of about 0·08 or 0·007 are obtained according to the method adopted in extrapolating the NR curve backward.

Group IV. Citric acid and alcohol production of Penicillium divaricatum *and* P. sanguifluus

On the Chrząszcz-Tiukow hypothesis of citric acid production every molecule of citric acid is derived from 3 molecules of ethyl alcohol. Hence if this hypothesis is correct it should be found that the alcohol output in citric acid producing species of moulds is sufficiently great to account for the total amount of citric acid produced. *P. divaricatum* and *P. sanguifluus* were chosen to test this hypothesis.

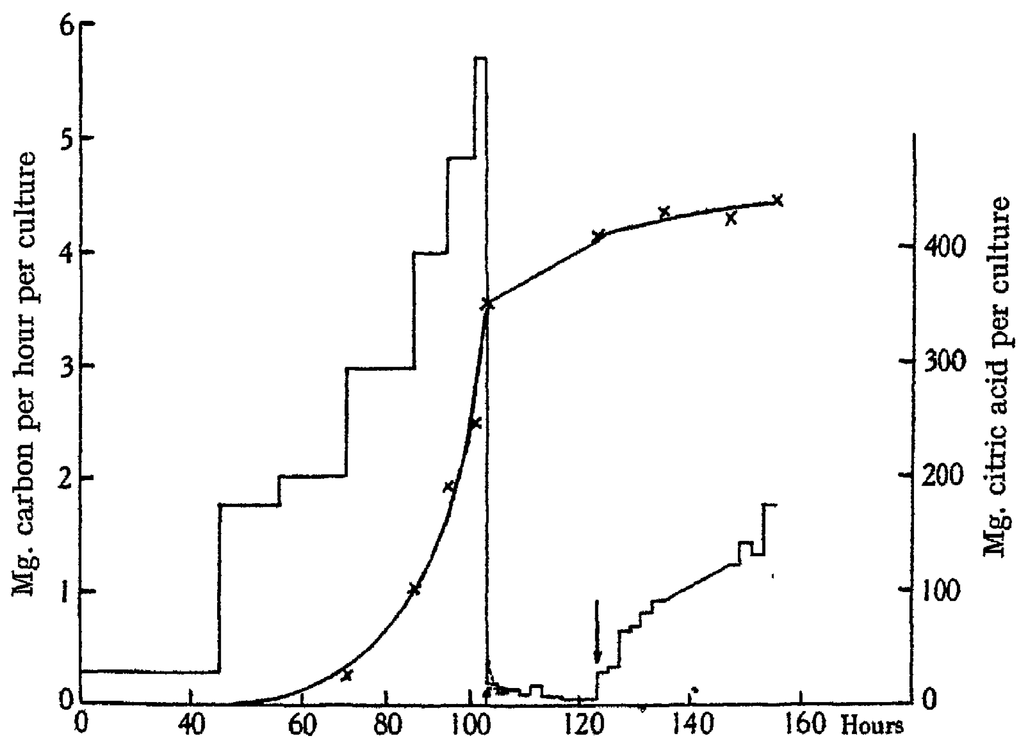

Fig. 7. Experiment G 1. *P. divaricatum.*

Exp. G 1. *P. divaricatum* was grown on 200 c.c. of 5% glucose with the inorganic salts of Czapek's solution in a 500 c.c. flask. By means of a 1 c.c. pipette, samples of the culture solution were taken periodically for titration and thus the rate of citric acid production could be estimated. At the 150th hr., when the rate of citric acid production appeared to be at its greatest, the fungus was put into nitrogen and a continuous stream of this gas was passed through the culture vessel at the rate of 1 l./hr. The carbon-dioxide output was measured every 4 hr. In Fig. 7 the course of respiration in air and

nitrogen is given by the stepped graph and the smooth curve gives the total citric acid carbon in solution. Following the method of the previous section in extrapolating back the nitrogen curve, the initial NR is 0·4 or 0·15 mg. C/hr., depending on the construction used. Since equimolecular amounts of alcohol and carbon dioxide are produced in nitrogen, the rate of alcohol carbon production is 0·8 or 0·3 mg. C/hr. The rate of citric acid carbon production at the 150th hr. obtained from the gradient of the smooth curve is 6·1 mg. C/hr. which is thus seen to be far in excess of the rate of alcohol production. The dry weight of the fungus was 0·51 g. so that 0·861 g. citric acid/g. dry weight of fungus was produced in 150 hr.

Exp. G2. Most investigators of the acid metabolism of the moulds have added calcium carbonate to the culture solution. The effect of this is to prevent the resynthesis of the acids into other products and thus the maximum acid yield may be obtained. A culture of *P. divaricatum* similar to that in Exp. G1 but containing 1 g. calcium carbonate was allowed to grow for 7 days. On analysis the total amount of citric acid in the form of calcium citrate in the culture solution was 1·02 g. and the dry weight of fungus 0·25 g. giving an output of 2·55 g. citric acid/g. dry weight of fungus. Compared with the yield obtained in the previous experiment, this output is three times as great. Hence it may be stated that the zymasic activity is quite inadequate to account for the maximum yields of citric acid obtained in cultures of *P. divaricatum*.

Exp. G3. *P. sanguifluus* was used as the citric acid producing species. The data is given graphically in Fig. 8. In the first 120 hr. 0·080 g. citric acid was produced by the fungus. The rate of citric acid carbon formation at the 120th hr. is 1·1 mg. C/hr. The initial NR may be taken to be 0·5 or 0·06 mg. C/hr., depending on the construction used in extrapolating back the nitrogen curve. In terms of alcohol carbon these rates would be 1·0 or 0·12 mg. C/hr.

Hence the higher rate of alcohol carbon production is approximately equal to the rate of citric acid carbon production. The dry weight of the fungus is 0·96 g. and the total citric acid content of the culture/g. dry weight of fungus at the 120th hr. is 0·225 g. This is, however, the net citric acid yield and does not take account of that converted into other products.

Exp. G4. To obtain the maximum yield of citric acid *P. sanguifluus* was grown in the presence of calcium carbonate as in Exp. G2. After 7 days' growth the culture solution was analysed for calcium citrate. The amount of citric acid produced was 0·24 g. and the dry

weight of fungus 0·10 g., giving a citric acid production of 2·4 g. citric acid/g. dry weight of fungus. Without calcium carbonate 0·225 g. citric acid/g. was produced in 120 hr.: with calcium carbonate

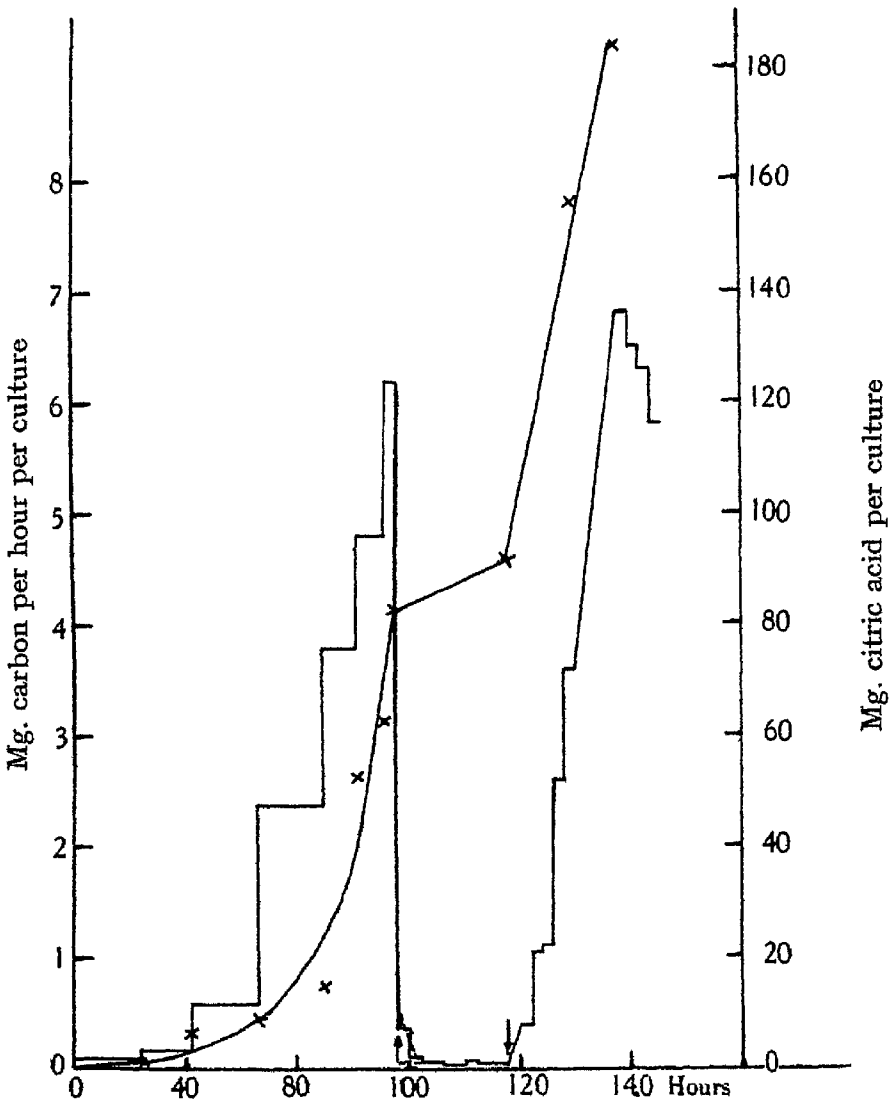

Fig. 8. Experiment G 3. *P. sanguifluus.*

2·4 g. citric acid/g. was produced in 165 hr., over ten times as much. When the maximum yields of citric acid are considered the zymasic activity of *P. divaricatum* and *P. sanguifluus* is insufficient to account for the total amounts of citric acid produced.

Discussion

The rate of alcohol and carbon-dioxide production in nitrogen may perhaps be legitimately taken as a measure of the quantity of "zymase", and if this be accepted it is noted that different species

of fungus possess strikingly different zymase contents. It is noteworthy that in spite of the great differences in zymasic activity, all the anaerobic carbon-dioxide output in all species examined can be accounted for as due to the activity of the zymase system of the fungus. In other words great differences are found in anaerobic respiratory rates of the various species examined. Comparison of the air and nitrogen respiration reveals the most surprising diversity of behaviour in different species. It seems highly probable that in a number of species of *Aspergillus* and *Penicillium* the ratio NR/OR is less than 0·33. Values of 0·33 would perhaps find an explanation in the complete conversion of all products of zymase glycolysis to carbon dioxide and values above 0·33 are explicable in terms of anabolic resynthesis of part of the products of zymasis. Values below 0·33 require either revision of the view that the whole of the aerobic respiration can be accounted for by sugar zymasis or special pleading. It might be pleaded that an inactivation of the preglycolytic stage of the sugar supply mechanism is brought about in the absence of oxygen. If this is the case the inactivation rate in the fungi examined here is enormously greater than the rate of change of activity of the preglycolytic phase as deduced for apples. Alternatively, and it seems to us more reasonable, it may be postulated that zymasis plays a relatively small part in the aerobic breakdown of sugar in those types such as *Aspergillus niger* and *Penicillium sanguifluus*. In those cases the greater part of the aerobic carbon-dioxide output may be regarded as being formed in reactions which have as their starting point the oxidative conversion of glucose to gluconic acid and the further breakdown of the latter by channels as yet only partially explored. This type of glycolysis would cease on removal of oxygen and zymasis (which may be assumed to be also occurring in air but affecting much less sugar than the glucose oxidase) becomes the sole source of glycolysis. Such a system is, of course, not susceptible to the treatment applied by Blackman to apples since the glucose oxidase mechanism is itself affected by the oxygen tension. These postulates are in agreement with several lines of research. They agree closely with Müller's finding that in cultures of *Aspergillus niger* growing on glucose a direct oxidation of the glucose molecule to gluconic acid takes place by means of the enzyme glucose oxidase. According to Müller, zymase plays a very small part in the breakdown of glucose by *A. niger* and sugar breakdown is due primarily to glucose oxidase. They are, however, in very marked disagreement with the Chrząszcz-Tiukow hypothesis of citric acid formation. According to this

hypothesis, citric acid formation from glucose takes place in two stages. First, glucose is broken down to alcohol by zymase,

Glucose → methyl glyoxal → pyruvic acid → acetaldehyde → alcohol.

Then follows a process in which oxygen takes part and citric acid is synthesized from acetaldehyde or alcohol. Since the only source of citric acid would be acetaldehyde or alcohol, and since these substances do not accumulate in the culture solution, the Chrząszcz-Tiukow hypothesis demands that the rate of citric acid-carbon formation should not exceed the rate of alcohol-carbon formation. The rate of citric acid-carbon formation can easily be determined and the rate of alcohol-carbon formation may be taken to be twice the rate of anaerobic carbon dioxide-carbon formation at the same time. The data of group IV make it quite clear that this condition is not realized in the cases of *Penicillium divaricatum* and *P. sanguifluus* when the gross rate of citric acid formation is measured and not the net rate, i.e. the excess citric acid formation over citric acid resynthesis into other products.

Kostytschew's statement that peptone cultures are zymase-free suggested another point of attack on the problem of the "zymasic-origin" of citric acid. Our results, however, show that peptone cultures do in fact contain zymase though the quantity (on the basis of rate of alcohol production) seems to be smaller than in glucose cultures. This apparently smaller quantity, however, involves the problem of the basis to which quantities should be referred. The peptone cultures contain a smaller quantity of zymase/g. dry weight but it is not clear that dry weight is a suitable basis of reference (see Bennet-Clark & La Touche, 1935), and it must be conceded that there are no proper means for comparing quantitatively peptone cultures with sugar cultures. The impossibility of obtaining a zymase-free culture makes it impossible to produce citric acid in the complete absence of zymase, but the fact that in two species examined the rate of citric acid-carbon formation was in excess of the rate of alcohol carbon formation would seem to make the Chrząszcz-Tiukow theory of citric acid formation untenable.

Summary

1. Normal zymasic alcoholic fermentation is found to take place in cultures of the 8 species of *Aspergillus* and 4 species of *Penicillium* examined. No correspondence is found between zymasic activity and citric acid production.

2. Peptone cultures of *Aspergillus niger* are found to be capable of fermenting sugar in the absence of oxygen.

3. The course of carbon dioxide production during alternate periods in air and nitrogen is investigated.

4. When the maximum rates of citric acid formation in cultures of *Penicillium divaricatum* and *P. sanguifluus* supplied with calcium carbonate are considered, it is found that the rate of citric acid-carbon production is greater than the rate of alcohol-carbon formation in nitrogen.

In conclusion, the author wishes to express his thanks to Dr T. A. Bennet-Clark for his constant help and interest throughout this work.

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REVIEWS

Biological Effects of Radiation. Edited by BENJAMIN M. DUGGAR. 2 vols. 1342 pp. London: McGraw-Hill Publishing Company, Ltd. 1937. Price £3. 10s.

This collection of forty-three papers is a "by-product from one of the activities of a committee now known as the Committee on Radiation, in the Division of Biology and Agriculture, National Research Council" of the United States. It is intended to "represent the present status of scientific knowledge in as many as practicable of the aspects of radiation in relation to biological problems" and to have "the qualities of factual presentation, critical analysis, and systematization of method that are required for better orientation of the research or teaching biologist, the interested physicist or photochemist, and the qualified general reader". This we learn from the preface by the editor, Prof. Duggar.

Any such attempt to make a fresh cross-section of science to be presented to such a wide variety of readers is sure to meet with difficulties. There is the difficulty of selection of topics even when the field has been limited by the exclusion of applied radiology. For example, a 26-page paper on statistical treatment is included; a paper which makes no reference to the treatment of the problem of small samples. The question arises as to whether it is worth while to include such a short paper, for if the reader is not already familiar with what is presented it is perhaps better that he should go straight to one of the textbooks mentioned in the paper. If statistical treatment is discussed why is not other quantitative treatment of data included, particularly when in another paper we find "a graph in which concentration c is plotted against time t may be extrapolated within reasonable limits" (p. 267)? The paper in itself is clearly written and should be helpful, for as the author says "one not infrequently sees papers in which the observations have been given statistical treatment, but the constants have been derived because they are fashionable, since at no time have they been used for the development of a scientific argument". The 50-page paper on photochemistry (which has a misprint of l for I on p. 259) raises the same questions. This paper is so condensed as to run the risk of misleading a reader who requires such an introduction to the subject. In treating Lambert's Law and Beer's Law no mention is made of the fact that these laws apply only to those portions of the spectrum, usually very narrow, where k, the extinction coefficient, is essentially constant. In view of the quotation from the preface which we have given it is perhaps surprising to find the author of this paper showing confusion about the Blackman reaction in photosynthesis when he says "in the so-called Blackman region when at high intensity of illumination the photosynthesis is proportionately less...because the carbon dioxide material cannot diffuse rapidly enough into the cells".

It will perhaps be encouraging for the investigator struggling along in one branch to become acquainted with the chaotic state of other branches such as that revealed by Prof. S. C. Brooks who writes about "The Effect of Radiation on Venoms, etc." He says "the experiments *in vitro* suffer from lack of adequate quantitative technique; workers...have almost uniformly failed to use enough animals to give their results statistical validity".

Taking the book as a whole there is a real wealth of information covering a wide range of topics and, although it may not be the opinion of everyone that it is an ideal book, yet physicists, chemists and biologists who consult it will learn much of each other's activities and difficulties.

G. E. BRIGGS

The Plant Diseases of Great Britain: A Bibliography. Compiled and annotated by G. C. AINSWORTH. 9×6 in. xii+273 pp. London: Chapman and Hall. 1937. Price 15s.

As Dr Butler points out in the Foreword, the task undertaken by Dr Ainsworth of compiling and annotating a bibliography for the plant diseases of Great Britain was one which was happily conceived. This book contains annotated references to all important papers published in this country on the pathology of cultivated plants since the subject received attention at the hands of M. J. Berkeley, M. C. Cooke and other pioneers. In addition, valuable reference is made to work in other countries on diseases which also occur in Britain. All plant pathologists who do not keep a complete card-index of the literature on their subject will welcome this bibliography and will be under a debt of gratitude to Dr Ainsworth for the care with which he has compiled it. The book is very conveniently arranged, the diseases being listed under the cultivated plants which they affect. Unfortunately, a book of this kind is partly out-of-date almost as soon as it appears, so rapid is the output of papers in this field. With this bibliography before them, however, plant pathologists will be stimulated to maintain their references to current literature. The book is well indexed and is very clearly printed.

F. T. BROOKS

Plant Ecology. By HILDA DRABBLE. 9×6 in. Pp. 142, with 12 plates. Arnold. 1937. Price 7s. 6d.

Dr Drabble has written a book which meets a very clear long-standing need of advanced school botanists: Prof. Tansley's *Types of British Vegetation* has long been out of print and it was too detailed for school use, and no new text exists which incorporates the considerable amount of work published since 1911 on different kinds of vegetation in this country. Dr Drabble's book, in an elementary way, does do this, and for the first time the advanced school student can obtain not only descriptions of interesting vegetation types, but also some account of the ecological principles which they illustrate.

It was a useful idea to make the introductory part of the book concerned with the soil and the manifold relations of the plant to it. My own feeling is that this section is so condensed that it is too elementary for students who will read the rest of the book, but it does cover, if briefly, many topics essential in an introduction to ecological study.

The author has so far succeeded in her intention of writing a good school book, that it would be unfair to judge its contents by the standard of a professional ecologist. I feel, however, that before long the distinctive categories of "blanket-bog" or "raised-bog" should be recognized in this country, the term "heather-moor" or "cotton-grass moor" is quite inadequate for them. They cover large areas and are among the least spoiled of our vegetational types.

Both appearance and scientific value of the book are enhanced by 24 exceedingly good vegetational photographs by Prof. Salisbury: they represent a standard at which school (and professional) photographers might well aim.

A book such as this should encourage the work of those botanical educationalists, happily increasing in number, who teach the subject in the field and in relation to the living plant in its environment.

H. GODWIN

Zur Soziologie der Isoëtetalia. By MAX MOOR. Pp. 1–148, with 7 plates, 11 text-figures and 4 tables. *Beitr. zur geobot. Landesaufn. der Schweiz*. Heft 20. Price 6·50 fr.

The *Isoëtetalia* (Associations Ordnung) is divided into two Associations Verbände, the *Nannocyperion flavescentis* and the *Isoëtion*. The latter is essentially Mediterranean and is spread over southern Spain and France and the north coast of Africa. The former is middle European and occupies central and north Europe and the southern parts of England and Scandinavia. The characteristic plants of the Ordnung are of Mediterranean origin, and hence it is suggested that the *Nannocyperion* is younger than the *Isoëtion* and has wandered north after the retreat of the ice. An interesting idea is the conception of the *Isoëtion* and the *Nannocyperion* as vicarious Verbände, whilst there are a number of species in both which are regarded as forming vicarious pairs. The two Verbände are geographically separated by the Pyrenees and the Swiss Alps. They both colonize in their respective regions the same kind of habitats, namely banks and edges of ponds and lakes that are periodically flooded, ditches in grain fields, wet woods and reed swamps where there is stagnant water. In general aspect they are Therophyte communities and so are usually to be found colonizing bare ground and their appearance is often limited by the length of time the ground is free from water and available for colonization. The plants composing the communities are generally small, 5–10 cm. high, and the associations only occupy relatively small areas.

The history of the *Isoëtetalia* Ordnung is given and then the two Verbände are described. The *Nannocyperion flavescentis* is divided into five associations and the *Isoëtion* into seven associations—four in south Europe and three in North Africa. The bulk of the paper is concerned with a detailed description of each of these associations, including an account of their geographic spread.

For English readers the subject-matter is complicated by the use of the continental system of nomenclature, e.g. the Association is equivalent to the English and American Consociation, whilst the Ordnung is equivalent to the Anglo-American Association.

This paper fills a definite gap in our knowledge of the smaller and somewhat more insignificant communities of Europe. There is a good bibliography at the end.

V. J. CHAPMAN

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